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ORIGINAL ARTICLE

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Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells

Abstract We investigated the expression levels of several genes related to cell proliferation in human mesenchymal stem cells (hMSCs) during in vitro culture for use in clinical applications. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. The proliferation rate of hMSCs gradually decreased and marked changes in hMSC morphology were not observed in 3 months of in vitro culture. The mRNA expressions of TGFβ1, TGFβ2, and TGFβ receptor type I (TGFβRI) in hMSCs increased with the length of cell culture. There had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage from the primary culture (at about 3 months). The mRNA expression of Smad3 increased, but those of c-myc and nucleostemin decreased with the length of hMSC in vitro culture. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells. In conclusion, hMSCs derived from bone marrow seldom underwent spontaneous transformation during 1–2 months of in vitro culture for use in clinical applications. In hMSCs as well as in epithelial cells, growth might be controlled by the TGFβ family signaling.

Key words Stem cells · Cell proliferation · TGFβ signaling · TGFβ receptors

Introduction

Several recent studies demonstrated the potential of bioengineering using somatic stem cells in regenerative medicine.[1,2] Bone marrow includes both mesenchymal and hematopoietic stem cells. Adult human mesenchymal stem cells (hMSCs) derived from bone marrow have the pluripotency to differentiate into cells of mesodermal origin, e.g., bone, cartilage, adipose, and muscle cells.[1–5] Moreover hMSCs also have the capacity to differentiate into myocytes,[6,7] hepatocytes,[1,8] and neural cells.[3] In addition, because they are comparatively easy to expand ex vivo, hMSs have many potential clinical applications, not only in the field of orthopedic surgery but also for the treatment of cardiac infarction, cirrhosis, and diabetes. On the other hand, stem cells possess a self-renewal capability similar to that of cancer cells.[9] Recently Rubio et al.[10] reported spontaneous transformation of human adult stem cells derived from adipose tissue in long-term (4–5 months) in vitro culture. In practice, if hMSCs are to be used for clinical applications and tissue-engineered medical devices, they have to be expanded in vitro for about 1–2 months. The proliferation ability and the gene expression profile of hMSCs, however, might change during in vitro culture. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. TGFβ is a multifunctional protein that regulates cellular proliferation, differentiation, apoptosis, development, extracellular matrix formation, immunosuppression, and tumorigenesis. In humans, three TGFβ isomers have been identified: β1, β2, and β3. TGFβ signals through three high-affinity cell surface receptors: TGFβ type I (TGFβRI), type II (TGFβRII), and type III (TGFβRIII) receptors. TGFβRI and TGFβRII are serine-tyrosine kinases. TGFβRIII is known to be a betaglycan.[11] TGFβs are first bound to TGFβRII and TGFβRIII.[12] It has been considered that TGFβRIII regulates access to TGFβRII,[12–14] and then TGFβ signal transduction in the cellular pathway is started through stimulation of TGFβRI by TGFβRII. After that, activated TGFβRI phosphorylates Smad2 or Smad3, which are receptor-regulated Smads (R-Smad) activated by TGFβ and activin.[15,16] After Smad4, which is a common mediator Smad (C-Smad), is connected to phosphorylated R-Smads, the complex is transported to the cell nucleus and influences the transcription activity of TGFβ-dependent genes.[15,16] c-myc, which is one of the

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TGFβ-dependent genes, is regarded as an oncogene and regulates cellular proliferation. In the present study, therefore, we investigated whether the gene expression levels of three TGFβ isomers (TGFβ1, TGFβ2, and TGFβ3) and their receptors (TGFβRI, TGFβRII, and TGFβRIII), Smad3 and c-myc were changed in hMSCs during in vitro culture.

Wnt-8B is related to cell self-renewal and tumorigenesis,[9] and Wnt proteins can act as stem cell growth factors.[17] Wnt signaling activates the genes that promote proliferation (c-myc and others) by accumulating β-catenin in some kinds of stem cells and cancer cells.[9] Nucleostemin is involved in proliferation in both stem cells and cancer cells.[18] Therefore we also investigated the gene expression levels of Wnt-8B and nucleostemin in hMSCs.

In addition to investigating the expression of these genes relating to cellular proliferation in hMSCs during in vitro culture, we compared them with those in two kinds of cancer cell lines, HeLa S3 (a human cervical cancer cell line) and HepG2 (a human hepatoma cell line).

Materials and methods

Cell culture. Human mesenchymal stem cells (hMSCs) derived from bone marrow were purchased from Cambrex Bio Science (Walkersville, MD, USA). Their donor was an African American woman aged 19 years. The cells that we obtained from Cambrex Bio Science were second-passage cells. The hMSCs were cultured in mesenchymal stem cell basal medium (MSCBM; Cambrex Bio Science) supplemented with mesenchymal cell growth supplement (MCGS; Cambrex Bio Science), L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C under a 5% CO_2 atmosphere. The cells were seeded at a density of 6000 cells/cm^2 and were subcultured when they were just subconfluent (approximately 90% confluent) up to the 10th passage, corresponding to the 12th passage from when the hMSCs were collected from the donor. The human cervical carcinoma cell line HeLa S3 (JCRB Cell Bank, Osaka, Japan) was cultured using Ham's F-12 culture medium (Dainippon Pharmaceutical, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY, USA) and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The human hepatoma cell line HepG2 (Riken Bioresource Center, Tsukuba, Japan) was cultured using minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1 mM nonessential amino acids (NEAA) (Invitrogen), 10% FBS (Intergen), and 100 U/ml penicillin-streptomycin (Invitrogen).

Preparation of total RNA. Because the purchased hMSCs had been expanded in the manufacturing process as described above, we express the 1st passage of the hMSCs in this study as the 3rd from the primary culture. For quantitative real time-polymerase chain reaction (RT-PCR), total RNA was extracted from hMSC cultures during the 3rd, 5th, 7th, and 12th passages from the donor with Isogen (Nippon Gene, Toyama, Japan). Total RNA was also extracted from HeLa S3 and HepG2 cells once only with Isogen (Nippon Gene).

Quantitative RT-PCR. RNA was then reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Basel, Switzerland). Primers and annealing temperatures for the c-myc oncogene, nucleostemin, Wnt-8B, transforming growth factor (TGF)β3, and TGFβRIII are summarized in Table 1. Amplifications were carried out for 10 s at 95°C, for 15 s at each annealing temperature, and for 12 s at 72°C for 40 cycles. Amplifications of TGFβ1, TGFβ2, TGFβRI, TGFβRII, and Smad3, plus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, were performed using Light Cycler Primer Sets (Roche Diagnostics). PCR was performed in Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) in a Roche Light Cycler (software version 4.0).

Statistical analysis. All results are shown as means ± SD. The significance of the differences in mean values was evaluated by Student's *t* test.

Table 1. Primers and annealing temperatures used for real-time PCR

Gene name	GenBank accession number	Primer orientation	Nucleotide sequence	Starting sequence position	Size for the PCR amplicon(bp)	Annealing temp. (°C)
c-myc	V00568	Forward	5′- GCG AAC ACA CAA CGT C -3′	1626	315	50
		Reverse	5′- CAA GTT CAT AGG TGA TTG CT -3′	1940		
nucleostemin	X91940	Forward	5′- CCA TTC GGG TTG GAG TAA -3′	782	284	50
		Reverse	5′- CTG TCG AGC ATC AGC C -3′	1065		
Wnt-8B	NM_014366	Forward	5′- AGT GAC AAT GTG GGC T -3′	331	244	60
		Reverse	5′- CGT GGT ACT TCT CCT TCA G -3′	574		
TGFβ3	NM_003239	Forward	5′- AAA CAC CGA GTC GGA A -3′	535	284	60
		Reverse	5′- TGC CAC CGA TAT AGC G -3′	818		
TGFβRIII	NM_003243	Forward	5′- TCC CTA TCC CGC AAG C -3′	2369	189	60
		Reverse	5′- AGA TTA TCG AGG CGT CC -3′	2557		

PCR, polymerase chain reaction; TGFβ3, transfroming growth factor β3; TGFβRIII, TGFβ recepter type III

Results

The proliferation rate of hMSCs decreased with the length of in vitro culture (Fig. 1). The effects of the in vitro culture term on hMSC proliferation and the mRNA expressions of three TGFβ isomers (TGFβ1, β2, β3) and their receptors type I, II, and III (TGFβRI, RII, RIII) in hMSCs were investigated (Fig. 2). The mRNA expressions of TGFβ1, TGFβ2, and TGFβRI increased with the length of cell culture (Fig. 2A,B,D), but there had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage (at about 3 months) (Fig. 2C,E,F). In addition, the mRNA expression of Smad3, which is one of the R-Smads activated by TGFβ and activin, in hMSCs was investigated. The mRNA expression of Smad3 decreased in the 5th and 7th passages of hMSCs but increased in the 12th passage (Fig. 3). The mRNA expressions of c-myc in hMSCs were higher in the 5th and 7th passages than in the 3rd and 12th passages (Fig. 4A). The mRNA expressions of nucleostemin in hMSCs decreased with the length of cell culture (Fig. 4B).

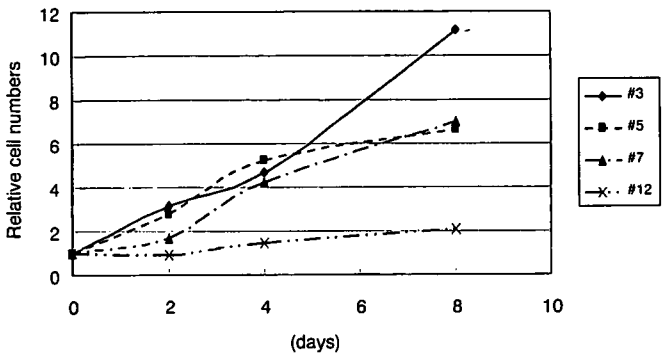


Fig. 1. Proliferation of human mesenchymal stem cells (hMSCs) in the 3rd, 5th, 7th, and 12th passages. hMSCs were seeded at 1.7×10^5 cells/F 60-mm dish (6000 cells/cm^2), and cells were counted after 2, 4, and 8 days. The initial cell number (0 days) is expressed as 1, and the other cell numbers (2, 4, and 8 days) are expressed relative to that of day 0. $n = 3$

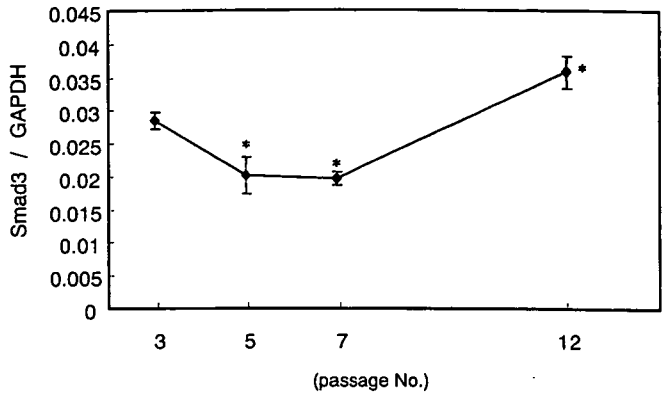
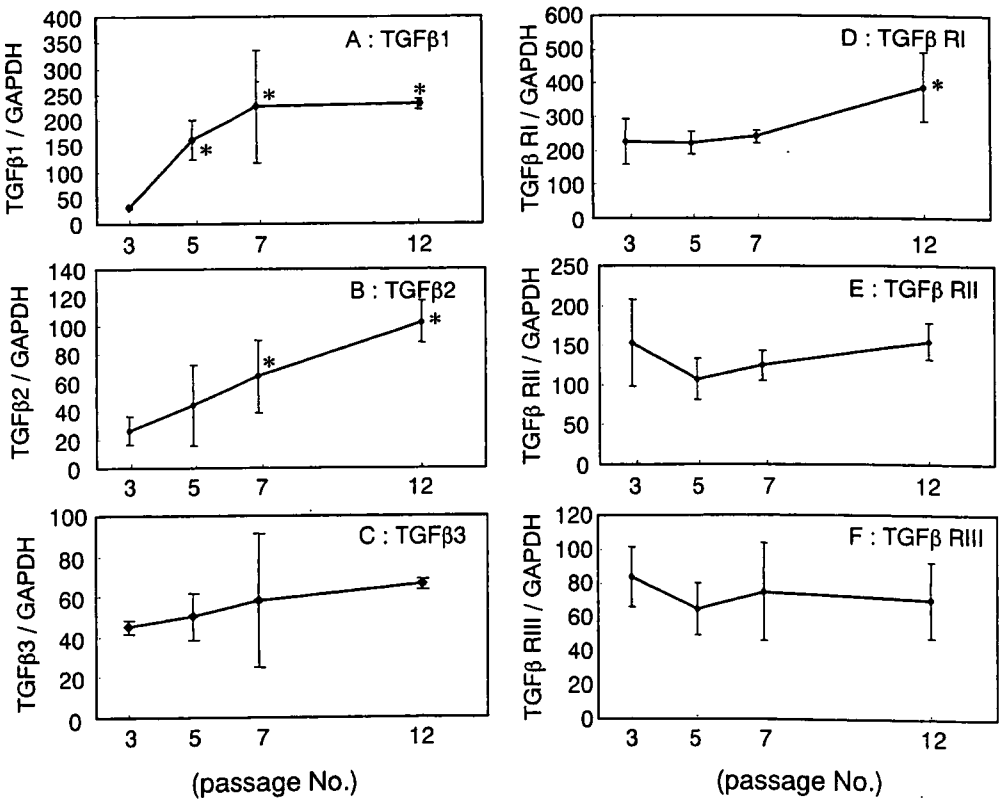


Fig. 3. Effect of in vitro culture length on mRNA expression of Smad3 in hMSCs. The expression of Smad3 relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages was investigated by quantitative RT-PCR. Mean values with SDs are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)

Fig. 2. Effect of in vitro culture length on mRNA expressions of transforming growth factor β1 (*TGFβ1*) (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβ receptor type I (*TGFβRI*) (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSCs. Expressions of the four genes, relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative real time-polymerase chain reaction (RT-PCR). Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)



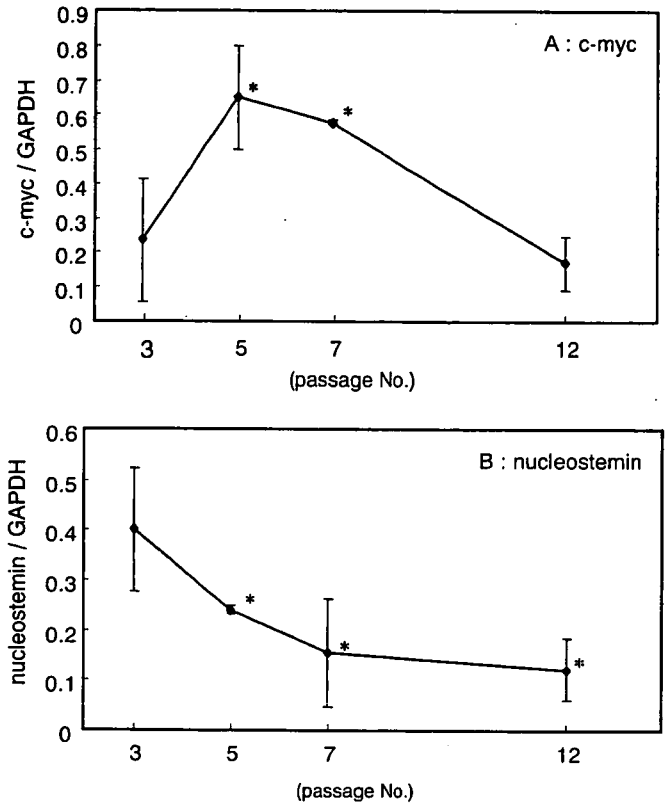


Fig. 4. Effect of in vitro culture length on the mRNA expressions of c-myc (**A**) and nucleostemin (**B**) in hMSCs. Expressions of the two genes relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)

The mRNA expressions of TGFβs and TGFβ receptors in hMSCs of the fifth passage were compared with those of two kinds of cancer cells (HeLa S3 and HepG2) (Fig. 5). TGFβ1 mRNA levels in hMSCs and HepG2 cells were significantly higher than those in HeLa S3 cells (Fig. 5A). The mRNA expressions of TGFβ2, TGFβ3, TGFβRI, and TGFβRII in hMSCs were significantly higher than those in the cancer cells (HeLa S3 and HepG2) (Fig. 5B,C,D,E). TGFβRIII mRNA expression in hMSCs was significantly higher than that in HeLa S3, but lower than that in HepG2 (Fig. 5F). The expressions of several genes affecting cellular proliferation in all three cells were also investigated. The mRNA expressions of c-myc oncogene and nucleostemin in the cancer cells (HeLa S3 and HepG2) were significantly higher than those in hMSCs (Fig. 6A and B). Wnt-8B mRNA was expressed in the cancer cells (HeLa S3 and HepG2), but not in hMSCs (Fig. 6C). Wnt-8B mRNA was not expressed in any passage numbers of hMSCs (data not shown).

Discussion

In this study, we investigated the changes of gene expression profiles during in vitro culture of hMSCs to evaluate their safety for use in clinical applications and tissue-engineered medical devices. First, the time dependency of the growth speed of hMSCs derived from bone marrow up to the 12th passage (at about 3 months) was investigated. The proliferation rate of hMSCs decreased by degrees during 3 months of in vitro culture (Fig. 1). No marked changes of hMSC morphology in 3 months of in vitro culture were

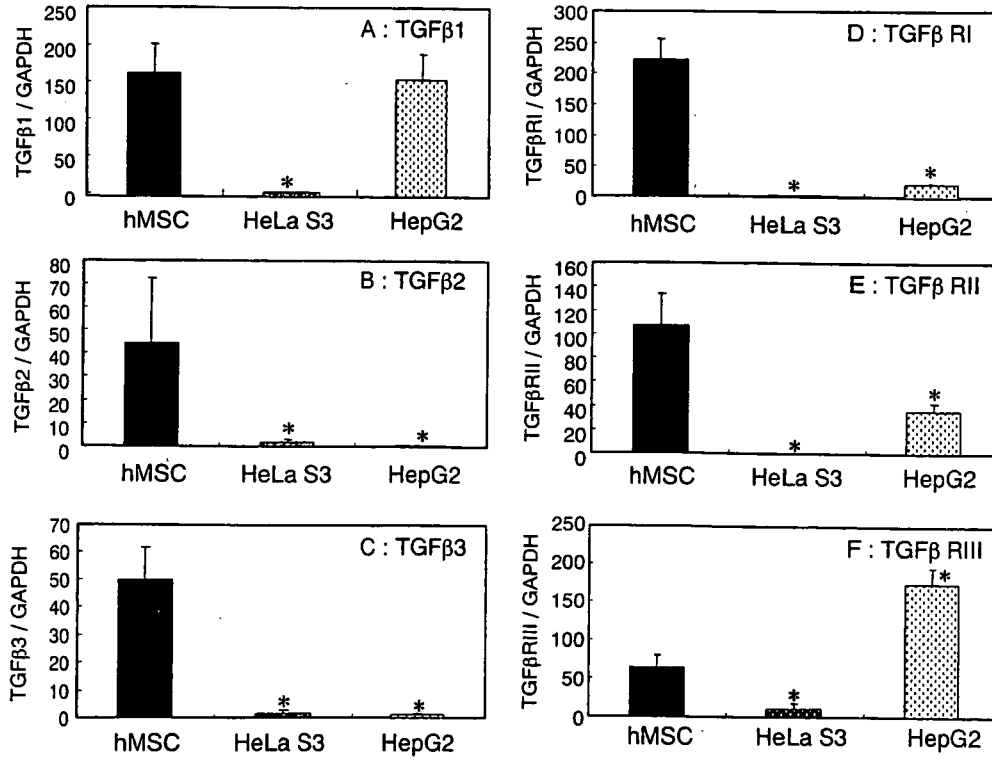


Fig. 5. mRNA expressions of TGFβ1 (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβRI (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSC, HeLa S3, and HepG2 cells. The expressions of the four genes relative to GAPDH in confluent cultures of hMSCs, HeLa S3, and HepG2 were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences from hMSCs (*$P < 0.05$)

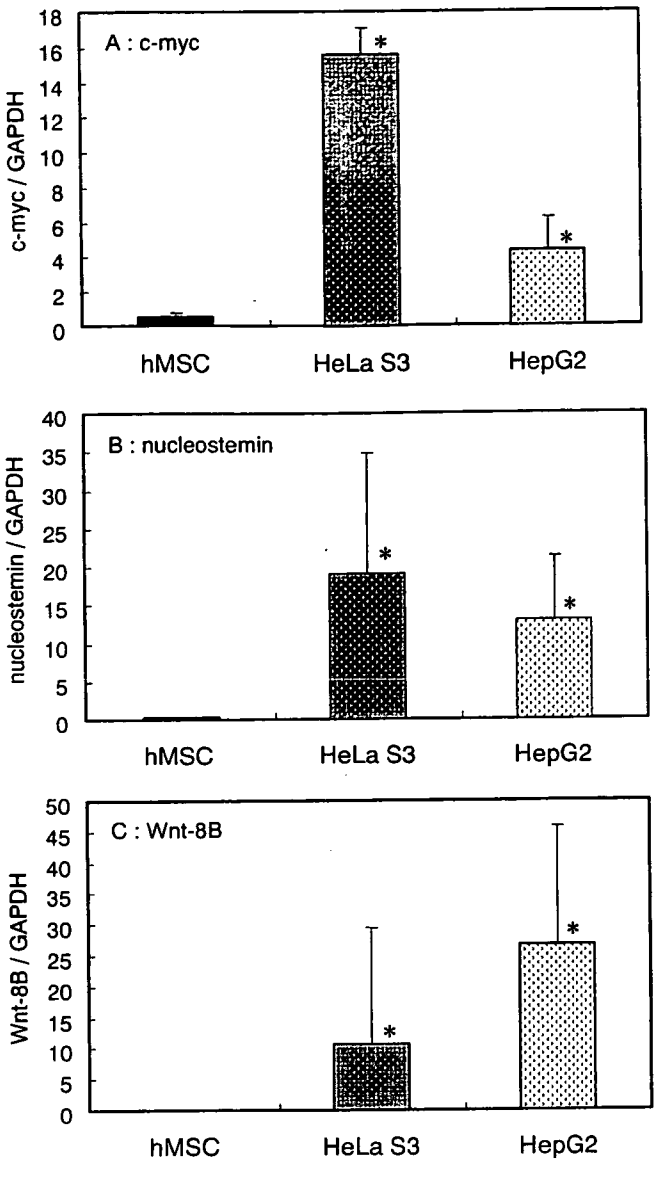


Fig. 6. mRNA expressions of c-myc (**A**), nucleostemin (**B**), and Wnt-8B (**C**) in hMSC, HeLa S3, and HepG2 cells. The expressions of the three genes relative to GAPDH in confluent cultures of hMSC, HeLa S3, and HepG2 cells were investigated by quantitative RT-PCR. Mean values with standard deviations from three independent experiments are presented. *Asterisks* denote statistically significant differences from hMSCs ($^*P < 0.05$)

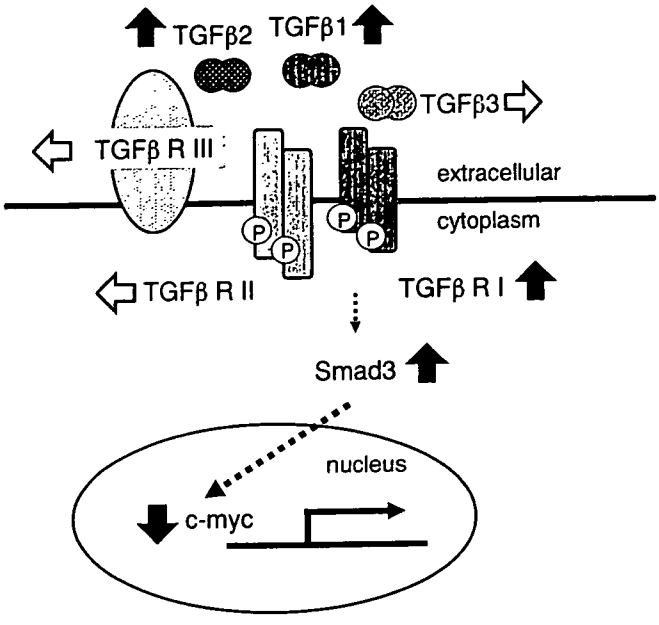


Fig. 7. Changes in the expressions of TGFβ signaling genes during hMSC in vitro culture for 3 months. The *dotted arrows* indicate the TGFβ signal pathway. *White arrows*, no changes; *black arrows*, up or down changes

observed. Several hMSCs derived from other donors' bone marrow did not undergo extraordinary proliferation either (data not shown). Adult stem cells have a self-renewal ability and undergo multilineage differentiation to maintain adult tissues.[9] In this study, however, hMSCs had more limited proliferative potential in in vitro culture. This phenomenon in hMSCs derived from bone marrow is the same result as that in hMSCs derived from adipose tissue reported by Rubio et al.[10] In addition, a decreasing cellular proliferation rate is often observed in several types of normal cells during in vitro culture. Consequently, these results suggest that hMSCs derived from bone marrow will seldom undergo spontaneous transformation during the 1–2 month period of in vitro culture necessary for use in clinical applications. But why does the proliferation of hMSCs decrease during in vitro culture? To focus on the proliferation mechanism of stem cells, we investigated whether the expressions of several genes related to cellular proliferation in hMSCs changed during in vitro culture. In the present study, we examined the expressions of TGFβs, their receptors, Smad3, c-myc, nucleostemin, and Wnt-8B. It has been proposed that the loss of TGFβRIII in renal cell carcinoma (RCC) is necessary for RCC carcinogenesis, and loss of TGFβRII leads to acquisition of the metastatic phenotype.[19] Therefore, the absence of changes in TGFβRII and TGFβRIII in hMSCs during in vitro culture might be important. The changes in mRNA expression levels during in vitro culture were different in each TGFβ isomer and receptor. TGFβ signal transduction in the cellular pathway is only possible through activation of TGFβRI. It was interesting that only TGFβRI mRNA expression increased with the length of cell culture among the three kinds of receptors (Fig. 2). The mRNA expressions of Smad3 increased (Fig. 3), but those of c-myc and nucleostemin decreased (Fig. 4) with the length of cell culture. We summarize the changes of TGFβ signaling gene expression during in vitro culture of hMSCs for 3 months in Fig. 7. TGFβ inhibits the growth of the many kinds of epithelial cells and hematopoietic, lymphoid, and endothelial cells.[20–23] In hMSCs as well as in the above-mentioned cells, hMSC growth might be controlled by TGFβ family signaling. As shown in Fig. 7, we hypothesized that the expressions of TGFβ1 and TGFβ2 in hMSCs increased during the period of in vitro culture, and then activated TGFβRI repressed the transcription of c-myc through Smad3; consequently, the cell cycle and cell growth might be arrested in hMSCs.

In addition, we compared the gene expression profiles of hMSCs with two kinds of cancer cell lines. One was HeLa S3 (a human cervical cancer cell line), which is markedly transformed, and the other was HepG2 (a human hepatoma cell line), which retains some hepatic functions. The mRNA expressions of TGFβs and their receptors in hMSCs were significantly higher than in the two types of cancer cells (HeLa S3 and HepG2) (Fig. 5). On the other hand, the mRNA expressions of c-myc and nucleostemin of the stem cells (hMSCs) were significantly lower than those of the two types of cancer cells (Fig. 6). Wnt signaling promotes self-renewal of hematopoietic, intestinal epithelial, and keratinocyte stem cells, among others;[9] however, Wnt-8B was not expressed in hMSCs derived from bone marrow (Fig. 6). These results suggest that expression of the genes that inhibit cellular proliferation and tumorigenesis were significantly higher and the genes that promote these processes were lower in hMSCs than in the cancer cells. Thus, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells.

Conclusion

In the present study, we confirmed that spontaneous transformation seldom occurred in hMSCs derived from bone marrow during 1–2 months of in vitro culture for use in clinical applications. In hMSCs, as in epithelial cells, growth might be controlled by TGFβ family signaling. During the period of in vitro culture of hMSCs, the expressions of TGFβ1 and TGFβ2 increased, and then activated TGFβRI repressed the transcription of c-myc through Smad3; consequently, the cell cycle and cell growth might have been arrested in hMSCs. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of the cancer cells.

Acknowledgments This work was partially supported by a grant for Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation, and Health and Labour Sciences research grants for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan.

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Effects of a biodegradable polymer synthesized with inorganic tin on the chondrogenesis of human articular chondrocytes

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Abstract: Recent study has shown that biodegradable polymers are attractive candidates for chondrocyte fixation and further transplantation in cartilage tissue engineering. Poly (glycolic acid) (PGA), a polymer of glycolic acid, is widely used in orthopedic applications as a biodegradable polymer. Organotin, lead, antimony, and zinc are catalysts commonly used in synthesizing PGA. Here, we investigated the biocompatibility of PGA, synthesized with and without inorganic tin as a catalyst in chondrogenesis of human articular chondrocytes in a micromass culture system. Significant enhancement of chondrocyte proliferation and expression of the collagen type II protein gene were observed in cultures treated with PGA synthesized with a tin catalyst. However, aggrecan gene expression was very similar to the control culture. Amount of collagen type II protein was also increased in the same group of cultured chondrocytes. In contrast, PGA without a catalyst caused overall inhibition of chondrogenesis. Despite several positive findings, extensive investigations are essential for the feasibility of this PGA(Sn) in future clinical practice. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 84–89, 2006

Key words: poly (glycolic acid); inorganic tin catalyst; human articular cartilage; chondrogenesis; micromass culture

INTRODUCTION

Different synthetic biodegradable polymers are currently gaining importance in the fields of biotechnology and tissue engineering. Recently, many studies have evaluated the potential of various natural bioabsorbable polymers such as collagen, [1,2] alginates, [3–5] fibrin, [6–8] and gelatin, [9] but synthetic biodegradable polymers in general offer advantages over natural materials. The primary advantages include the capacity to change the mechanical properties and degradation kinetics to suit various applications. Among the families of synthetic polymers, polyesters are used in a number of clinical applications.[10–12] Polyesters have also been used for development of tissue engineering applications, [13,14] particularly for bone tissue engineering.[15,12]

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The attraction of poly (glycolic acid) (PGA), one of the aliphatic polyesters, as a biodegradable polymer in medical applications is that its degradation product, glycolic acid, is a natural metabolite. Several studies have indicated that copolymers of glycolic acid caused promotion of nerve regeneration in a rat model, [16–18] and regeneration of an 80 mm nerve gap by an artificial nerve conduit made of PGA was also reported.[19] PGA can be synthesized using different catalysts. The common catalysts used include organotin, lead, antimony, and zinc. It was reported that inorganic and organic tin compounds present in the aqueous ecosystem have toxic effects and are capable of producing behavioral abnormalities in living organisms.[20,21] Organotin compounds are known to cause neurotoxicity, [22] cytotoxicity, [23] immunotoxicity, and genotoxicity [24] in human and other mammalian cells both *in vitro* and *in vivo*. Organotin compounds were also reported to decrease *in vitro* survival, proliferation, and differentiation of normal human B cells.[25] The dose effect of inorganic tin in rats suggests that the critical organ in inorganic tin toxicity is bone, [26] and disproportionate dwarfing syndrome, which severely affects the limbs but not the trunk, was observed in rats that had been injected with certain tin compounds.[27] As far as we know, no study yet has reported the chondrogenic

effects of PGA synthesized with and without an inorganic tin catalyst. In this study, the biocompatibility of PGA with and without a tin catalyst was investigated, using human articular chondrocytes (HAC) in a micromass culture system.

MATERIALS AND METHODS

Medium and polymers used for cell culture

Chondrocyte growth medium was obtained commercially from BioWhittaker (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (M_w = 1500) and without a catalyst (PGA) (M_w = 1100) were custom-made (TAKI chemicals, Kakogawa, Japan) and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical, St. Louis, MO, USA).

Cells and culture methods

Human articular chondrocytes (HAC) of the knee joint was commercially obtained from BioWhittaker. High-density micromass cultures were started by spotting 4×10^5 cells in 20 μL of medium onto Costar 24-well tissue culture microplates (Costar type 3526, Corning). After a 2 h attachment period at 37°C in a CO_2 incubator, culture medium (1 mL/well) was added to each well. Media were supplemented with DMSO (0.8 μL/mL), PGA, and PGA(Sn) (50 μg/mL). HAC cultured with DMSO was used as the control. The cultures were continued for 4 weeks with a medium change twice a week. At least four cultures were performed for each sample.

Cell proliferation study

Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[28] After the culture period, cells were fixed with 100% methanol at room temperature, followed by application of 0.1% crystal violet in methanol. After a proper wash, cells were again incubated in methanol; 100 μL from each well was transferred to a new 96-well plate, and the absorbance was measured at a wavelength of 590 nm, using an ELISA reader (Bio-Tek Instruments, Winooski, VT). Blank values were subtracted from experimental values to eliminate background readings.

Differentiation assay

Cell differentiation assay was performed by alcian blue (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[29] Following crystal violet staining, the cells were washed with methanol and then 3% acetic acid. Cultures were then stained with 1% (v/v) alcian blue in 3% acetic acid, pH 1.0. The cartilage proteoglycans were extracted with 4*M* guanidine hydrochloride (GH), and the bound dye was measured at wavelength of 600 nm, using an ELISA reader (Bio-Tek Instruments). Fresh 4*M* GH served as the blank. Blank values were subtracted from experimental values to eliminate background readings.

Analytical assays

Commercially available assay kits (collagen and glycosaminoglycan [GAG] assay kits, Biocolor, Newtownabbey, Northern Ireland) were used for the measurement of collagen and sulfated GAGs within the cultured cells, as previously described.[30]

Briefly, for the GAG assay, GAG was extracted from the cultured cells using a solvent system of 4*M* guanidine-HCl, 0.5*M* sodium acetate, pH 6, with 1 m*M* benzamidine-HCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 m*M* *N*-ethylmaleimide (NEM). Incubation was carried out at 4°C on an orbital shaker for a 12- to 20-h period. After the extraction, the samples were centrifuged, and blyscan dye reagent (composed of 1,9-dimethyl methylene blue in an organic buffer) was mixed with the supernatant. The GAG–dye complex was collected by centrifugation. The dye bound to the pellet was subsequently solubilized by mixing it with a dissociation reagent. The absorbance of the samples was measured at a wavelength of 656 nm, using a UV spectrophotometer. A calibration solution containing chondroitin-4 sulfate was used to obtain the standard curve for this experiment.

The total collagen concentration (acid- and pepsin-soluble fractions) of the cultured chondrocytes was also measured. The acid-soluble collagen was removed by adding 0.5*M* acetic acid to the cultured cells, followed by centrifugation. The remaining pepsin-soluble collagen was subsequently extracted from the cultured cells. A pepsin solution (1 mg/10 mg tissue sample; Sigma) was added to the cells, and they were incubated overnight at 37°C. Both the acid- and pepsin-soluble collagen samples were further separated for assay by mixing with Sircol dye reagent for 30 min in a mechanical shaker, and the collagen–dye complex was collected by centrifugation. The dye bound to the collagen pellet was solubilized with an alkaline reagent, and the absorbance of the samples was measured at a wavelength of 540 nm, using a UV spectrophotometer. A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment.

Real-time polymerase chain reaction

To detect the presence of collagen type II and aggrecan, single-stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT), using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently, real-time polymerase chain reaction (PCR) was done using a LightCycler system with LightCycler FastStart DNA Master SYBR Green I

(Roche Diagnostics, Penzberg, Germany). The LightCycler™- Primer set (Roche Diagnostics) was used for quantitative detection of the collagen type II and aggrecan genes, and also for quantitation of a housekeeping gene, Glyceraldehide-3-phosphate dehydrogenase (GAPDH), according to the manufacturer's instructions. An initial denaturation step at 95°C for 10 min was followed by amplification and extension steps for 35 cycles (95°C for 10 s, 68°C for 10 s, 72°C for 16 s) with final extension step at 58°C for 10 s. The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

Statistical study

Student's t tests were used to assess whether differences observed between the polymers treated and the control samples were statistically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD (standard deviation) except in figure 3. Four samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

RESULTS

Cell proliferation

Chondrocyte proliferation was quantified by crystal violet staining and expressed as a percentage of the average control value (Fig. 1). Cell proliferation was increased 1.8-fold ($p < 0.05$) in PGA(Sn)-treated cultures compared with that of the control culture, whereas cell proliferation in PGA-treated cultures was almost identical to the DMSO-treated control culture.

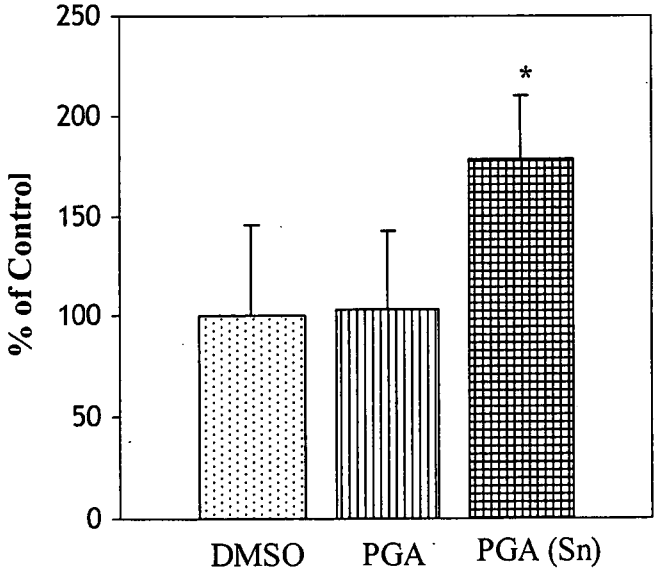

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

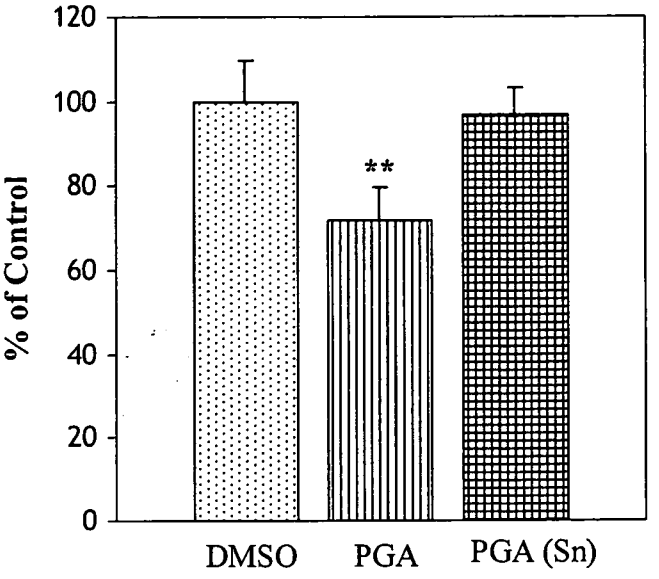

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

Cell differentiation

Chondrocyte differentiation was estimated by alcian blue staining and the amounts were expressed as a percentage of the average control value, which was calculated as 100%. Chondrocytes treated with PGA revealed a 0.71-fold ($p < 0.01$) decrease in cell differentiation compared with that of the control culture. At the same time, cultures treated with PGA(Sn) showed a slight, but nonsignificant, decrease in cell differentiation (Fig. 2).

Extracellular matrix gene expression

Extracellular matrix gene expression was quantitatively measured by real-time PCR. Here, compared with that of the control culture, the collagen type II gene was more strongly expressed ($p < 0.01$) in PGA(Sn) than in PGA-treated cultured chondrocytes [Fig. 3(A)]. Aggrecan gene expression was inhibited in the latter, but no difference was observed between the former and the control culture [Fig. 3(B)].

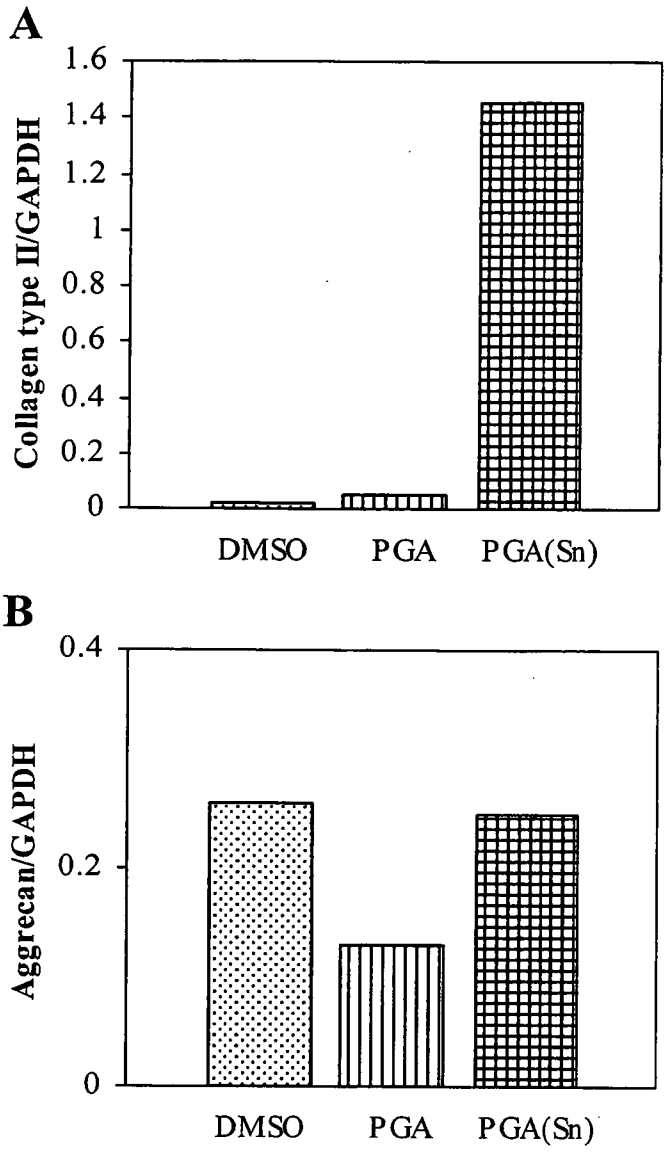

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between the PGA(Sn) and the control. All experiments were run in quadruplicate for two separate times.

Measurement of collagen type II protein

The amount of pepsin-soluble and cartilage-specific collagen type II protein was increased in both PGA and PGA(Sn) treated chondrocytes on comparing with that of the control culture(Fig. 4). However, this increase was more in the latter than in the former case.

Measurement of total collagen

Quantitative estimations of both acid- and pepsin-soluble total collagen revealed a decrease in PGA(Sn)-treated cultures compared with that of the control

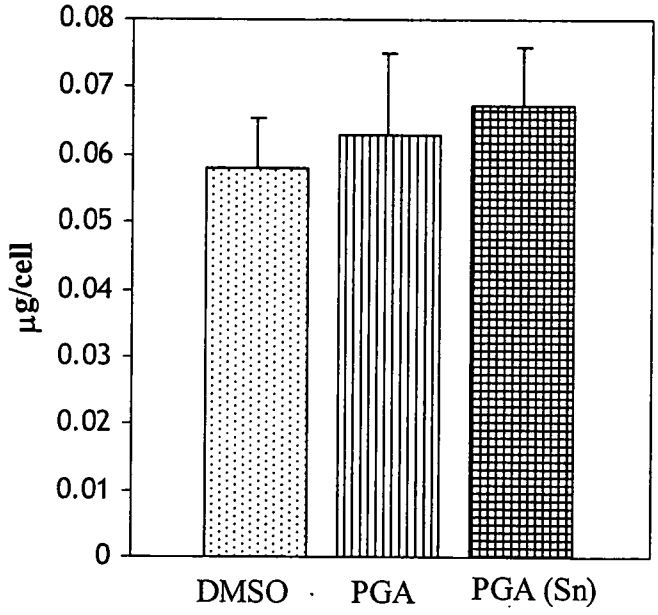

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

(Fig. 5). Simultaneously, there was a slight increase in the amount of total collagen in PGA-treated cultures compared with that of the control sample.

Estimation of sulfated glycosaminoglycan concentration

Evaluation of the amount of sulfated GAG showed a decrease in PGA(Sn)-treated cultured cells com-

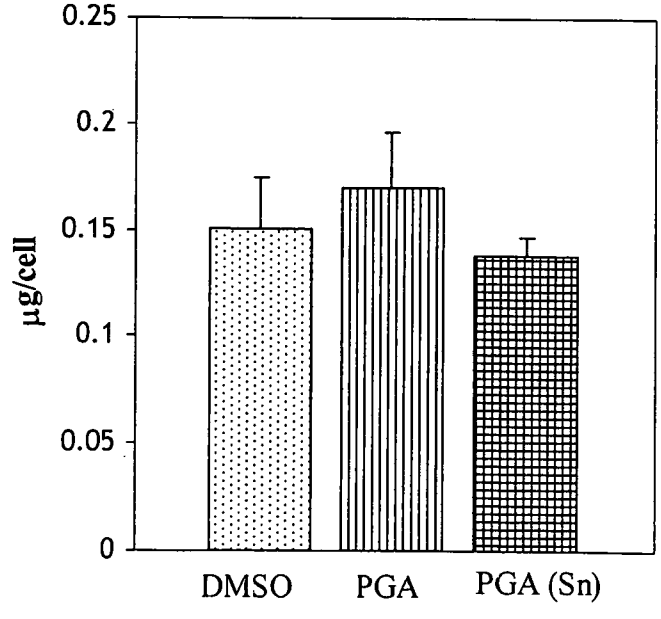

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

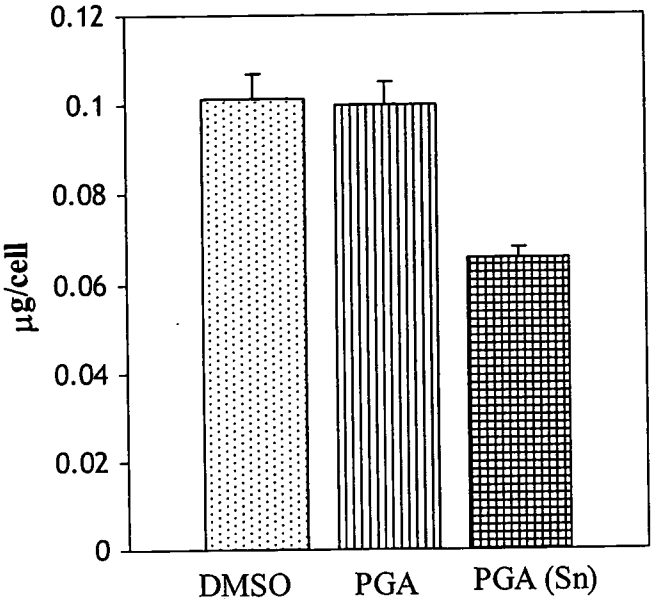

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

pared with that of the control (Fig. 6). However, in the same experiment, almost no difference in this amount was observed between the PGA-treated culture and the control.

DISCUSSION

Attempts to identify a perfectly biocompatible and biodegradable polymer have been ongoing over the past decade. An ideal biomaterial should fulfill its purpose satisfactorily and then biodegrade to obviate any risk of foreign body reaction.[31] Synthetic biodegradable polymers, especially those belonging to the polyester family, have played an important role in a number of tissue engineering efforts. PGA, an aliphatic polyester, can be degraded in two ways: by hydrolysis and by nonspecific esterases and carboxypeptidases, followed by either excretion in the urine or entrance into the tricarboxylic acid cycle.[32]

Several different catalysts, namely organotin, antimony, zinc, and lead, are used in the polymerization process to synthesize high molecular weight PGA. Different tin compounds were observed to produce general cytotoxic effects in rabbit articular cartilage in monolayer culture, [33] and bone is suggested to be the critical organ in inorganic tin toxicity in rats.[26] Therefore, in this study, we aspired to evaluate the chondrogenic effects of HAC with PGA synthesized with and without an inorganic tin catalyst, with the aim of clarifying the biocompatibility of inorganic tin as a catalyst for future clinical use.

It was reported that oral administration of certain tin compounds at specific concentrations exerted stimulatory effects on chondrocyte proliferation in the rat.[33] Consistent with this, the proliferation assay performed in our study also showed that HAC with PGA(Sn) had stimulatory effects on chondrocyte proliferation in micromass culture (Fig. 1). On the other hand, PGA neither stimulated nor inhibited the chondrocyte proliferation, and thus, inorganic tin as catalyst seemed to play a stimulatory role in HAC proliferation. In our experiment, PGA with inorganic tin as the catalyst caused almost no change in cell differentiation, but PGA-treated cultures did show a significant decrease when compared with that of the control (Fig. 2). Furthermore, quantitative estimation of extracellular matrix gene expression by real-time PCR confirmed that the cartilage-specific protein, collagen type II, was more strongly expressed in PGA(Sn)- than in PGA-treated cultured chondrocytes [Fig. 3(A)]. However, the expression of the aggrecan gene was inhibited in the PGA culture, but no difference was observed between the PGA(Sn) and the control cultures [Fig. 3(B)].

It was reported that oral administration of inorganic tin caused a decrease in the proliferation of chondrocytes, accompanied by suppression of DNA synthesis with subsequent inhibition in collagen synthesis in rat.[34] On the contrary, our results showed enhancement of proliferation, expression of the collagen type II gene, and amount of collagen type II protein by *in vitro* culture of HAC with PGA(Sn). We speculated that difference in the route of administration might be the cause of these diverse effects of inorganic tin compound. As mentioned earlier, monolayer culture of rabbit articular cartilage with tin compounds caused inhibition in the synthesis of core proteins, followed by a decrease in the synthesis of sulfated GAG.[33] In agreement with this result, our report also showed a decrease in the amount of sulfated GAG by culture of HAC with PGA(Sn). A study performed in our laboratory using HAC in a micromass culture system has already shown that PGA synthesized with organic tin catalyst caused a decrease in cell proliferation, but a significant increase in cell differentiation [29] and was completely contradictory to our present results. The molecular weight of PGA(Sn), and the type of tin product such as $SnCl_2$ and dibutyl tin were thought to be the key factor of different effects of chondrogenesis on HAC.

To the best of our knowledge, no other study has yet investigated the chondrogenic effects of PGA with inorganic tin as a catalyst, using HAC in a micromass culture system. This study is the first to show the biological action of inorganic tin as catalyst in PGA on human articular chondrogenesis in a micromass culture system. Our observation revealed that low concentration of inorganic tin when used in the polymer

of PGA showed enhancing effects of tin compounds on chondrocytes in comparison to without tin polymer because of increase in the permeability of inorganic tin under the presence of PGA. However, further study is required for the application of this PGA(Sn) in clinical practice.

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The effect of hyaluronic acid on insulin secretion in HIT-T15 cells through the enhancement of gap-junctional intercellular communications

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Abstract

The transplantation of bioartificial pancreas has the potential to restore endogenous insulin secretion in type I diabetes. The bioartificial pancreas is constructed in vitro from cells and a support matrix. Hyaluronic acid (HA) is an extremely ubiquitous polysaccharide of extracellular matrix in the body and plays various biological roles. It has been suggested that high molecular weight (HMW) HA increases in the function of gap-junctional intercellular communications (GJIC) and the expression of connexin-43 (Cx43). To determine whether the function of pancreatic β-cells is affected by gap junctions after HMW HA-treatment, we exposed HIT-T15, a clonal pancreatic β-cell line, in various concentrations of HA for 24 h, and then detected the insulin secretion and content, using an insulin assay kit by ELISA technique. The cellular functions of GJIC were assayed by dye-transfer method using the dye solution of Lucifer yellow. HA-treatment resulted in the enhancement of GJIC function, the increase of insulin release and insulin content. The results obtained in this study suggest that HA-coating increases the insulin secretion of HIT-T15 cells by the enhancement of Cx43-mediated GJIC. The results give useful information on design biocompatibility of HA when is used as a biomaterial for bioartificial pancreas.

Keywords: Hyaluronic acid; Gap-junctional intercellular communications; HIT-T15 cells; Insulin; Bioartificial pancreas

1. Introduction

Type I diabetes is caused by the autoimmune destruction of the β-cells. All patients with type I diabetes require daily insulin shots for the control of glucose levels. However, the insulin therapy cannot inhibit the development of serious chronic complications. The pancreas transplantation has been expected to be the most promising approach toward treating diabetes. The bioartificial pancreas is constructed in vitro from insulin-secreting cells or islets and a support matrix by a tissue engineering method. The frequently used matrix materials are alginate and agar [1,2]. Although bioartificial pancreatic constructs contain insulin-secreting cells entrapped in agar or alginate matrix implanted into the peritoneal cavity of the diabetic patient, mice, and dog, can restore normoglycemia and markedly abate diabetic symptoms, there are important questions in the structural integrity of support matrix, metabolic activity and viability of cells or islets, and late vascular thrombosis [1,2]. Therefore, the new matrix biomaterials, which mimic the functions of extracellular matrix (ECM), need to be researched.

Hyaluronic acid (HA) is an extremely ubiquitous member of the nonsulfated glycosaminoglycan ECM molecule family and is thought to play various biological roles particularly in growth, adhesion, proliferation, differentiation, and cell migration [3,4]. More importantly, the receptor for HA-mediated motility regulates gap-junction

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channel and connexin-43 (Cx43) expression by its actions on focal adhesions and the associated cytoskeleton [5]. In addition, Park and Tsuchiya [6] have reported that high molecular weight (HMW) HA-coating can enhance the function of gap-junctional intercellular communications (GJIC). The insulin secretion from pancreatic β-cells is a multicellular event depending on their interaction with neurotransmitters and numerous signal molecules carried by blood and also direct interactions between cell–cell and cell–matrix contacts by gap-junctional channels, which mediate exchanges of molecules smaller than 1000 Da, such as ions, small metabolites, and second messengers between adjacent cells. The latter interactions are thought to be crucial regulatory mechanisms of insulin secretion [7–9], and the pharmacological blockade of GJIC markedly decreases insulin release [8]. However, the effects of HMW HA as biomaterials of support matrix on functions of pancreatic β-cells and gap-junctional channel remain unclear.

In the present study, we investigated the effects of HMW HA on the function of GJIC, the expression of Cx43, insulin content, and insulin secretion using HIT-T15 cells in vitro. These results suggest that HMW HA can be used as the biomaterial for the development of a bioartificial pancreas: design biocompatibility of HA depends on the molecular-weight size of HA, and its application method and concentration.

2. Materials and methods

2.1. Materials

Lucifer yellow was purchased from Molecular Probes (Eugene, OR). HA (1680 kDa) and TetraColor ONE (WST-8) were supplied by Seikagaku Industries, Ltd. (Tokyo, Japan). ELISA insulin assay kit was obtained from Morinaga Seikagaku Co. (Yokohama, Japan). Bovine serum albumin (BSA) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Krebs–Ringer bicarbonate (KRB) buffer (pH 7.4), fetal bovine serum (FBS), and anti-Cx43 were purchased from Sigma Chemical Co. (St. Louis, MO). β-actin antibody was obtained from Cell Signaling Technology Inc. (Tokyo, Japan). Roswell Park Memorial Institute (RPMI) 1640 medium was from Nissui pharmaceutical Co. (Tokyo, Japan). All other chemicals used were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of media and culture dishes

The HA polysaccharide was dissolved in distilled water at a concentration of 4 mg/ml. Each of the 35-mm culture dish (Falcon 1008, Becton Dickinson) was coated at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. The HA-coated dishes were dried further under sterile air flow at room temperature for 12 h before use. In order to investigate the effect of HA-addition on the functions of HIT-T15 cells, different media were prepared at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. HA-treatment is performed to cells for 24 h.

2.3. Cells and cell culture

A hamster pancreatic β-cell line, HIT-T15 (HIT-T15 cells, Dainippon Pharmaceutical Co., Japan), was cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 IU penicillin-G and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The subculture cells were seeded at a density of $1.0–5.0 \times 10^5$ cells/ml in multiwell plates or culture dishes. When they reached more than 80% confluence, the cells were used for various studies. Throughout the cell growth period the culture media were replaced every 2 days.

2.4. Measurement of cell viability

To evaluate the affect of HMW HA on cell viability of HIT-T15 cells, HIT-T15 cells (1×10^5) were incubated into the various concentrations of HA-coated 24-well plates, or after the cells were seeded onto 24-well plates and pre-incubated in a 10% FBS/RPMI 1640 medium overnight, the medium was exchanged for 10% FBS/HA/RPMI 1640 medium prepared. After 24 h of HA-treatment, the cell viability was determined by the WST-8 reduction assay, according to the manufacturer's instructions. Control cells received fresh medium without HA.

2.5. Measurement of insulin release and insulin content

HIT-T15 cells were treated as described above. After pre-incubating for 30 min at 37 °C in KRB buffer, no glucose cells were stimulated for 60 min with 11.1 mM glucose in KRB buffer. The medium was collected, centrifuged for 5 min at 3000*g*, and the supernatant was frozen at −80 °C for insulin release assay. Cultures were then extracted for 24 h at 4 °C in acid-ethanol and the extracts also frozen for determination of insulin and protein content. Insulin was determined by ELISA insulin kit with rat insulin as standard, according to the manufacturer's instructions. Protein content was measured by the BCA protein assay reagent kit with albumin as standard (PIERCE). Values of secreted insulin were normalized to protein content.

2.6. Measurement of dye transfer

Gap junction-mediated communication between β-cells regulates the insulin secretion and insulin biosynthesis. Because HMW HA-coating increased the insulin release and insulin content but not HA-added, we tested whether the HA-coating increases the insulin secretion and insulin content have a relationship with gap junctions between HIT-T15 cells. HIT-T15 (5×10^5) cells were exposed to the HA-coated (0.1, 0.25, and 0.5 mg/dish) 35-mm glass coverslip (Ashland, MA) and incubated for 24 h to evaluate dye coupling using Lucifer yellow. The cells were rinsed with phosphate-buffered saline [PBS(+)] containing Ca^{2+}/Mg^{2+}, and 3 ml of PBS(+) containing 1% BSA and 10 mM HEPES (pH 7.4) were added to keep a sufficient pH stability under the microscope. The junctional coupling of HIT-T15 cells was determined by injecting Lucifer yellow into individual cells within monolayer clusters. Injections were performed on a phase-contrast microscope with InjectMan NI2 and microinjector FemtoJet (Eppendorf AG, Germany) using glass micropipette that were filled with a 4% solution of Lucifer yellow CH (MW 457.2) dissolved in 0.33 M lithium chloride, as previously described [11]. An injection pressure of 6.5 psi for 200 ms was used for each injection. The coupling extent was evaluated by counting dye-transferred cells at 2 min after microinjection. There was no leakage of injected dye into the medium.

2.7. Western blot analysis

HIT-T15 cells were grown into the various concentration of HA-coated 100-mm plastic dishes (0.1, 0.25, and 0.5 mg/dish) (FALCON 3003; Falcon) for 24 h, rinsed with Ca^{2+}/Mg^{2+}-free PBS(−) and then lysed in CelLytic™-M lysis/extraction reagent (Sigma). Protein content was measured by the BCA protein assay reagent kit (PIERCE). Samples of total extracts (20 μg protein/lane) were fractionated by eletrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The contents of the gels were transferred to PVDF membranes (Clear Blot Membrane-P). Membranes were saturated for 2 h at room temperature in Block Ace (Dainippon Pharmaceutical Co.,