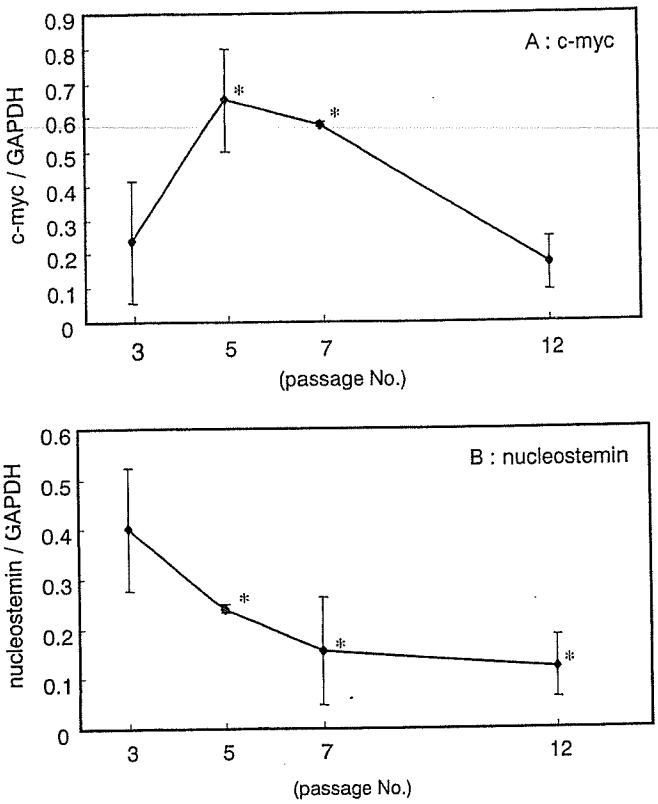


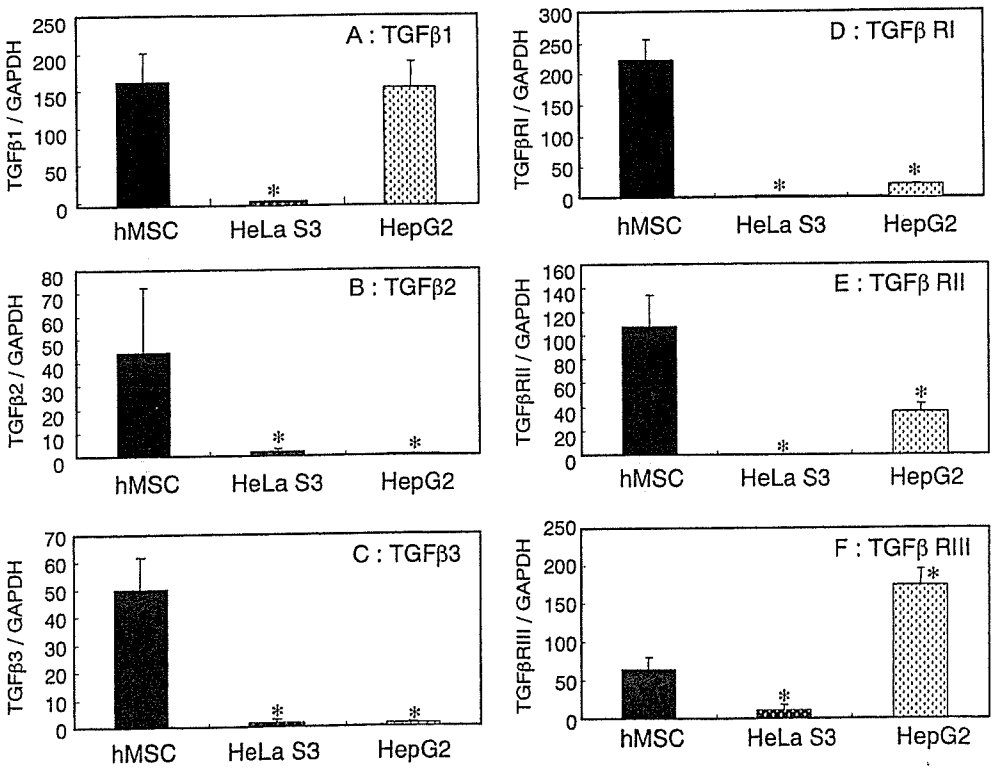
Fig. 4. Effect of in vitro culture length on the mRNA expressions of c-myc (**A**) and nucleostemin (**B**) in hMSCs. Expressions of the two genes relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative RT-PCR. Mean values with SDs from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



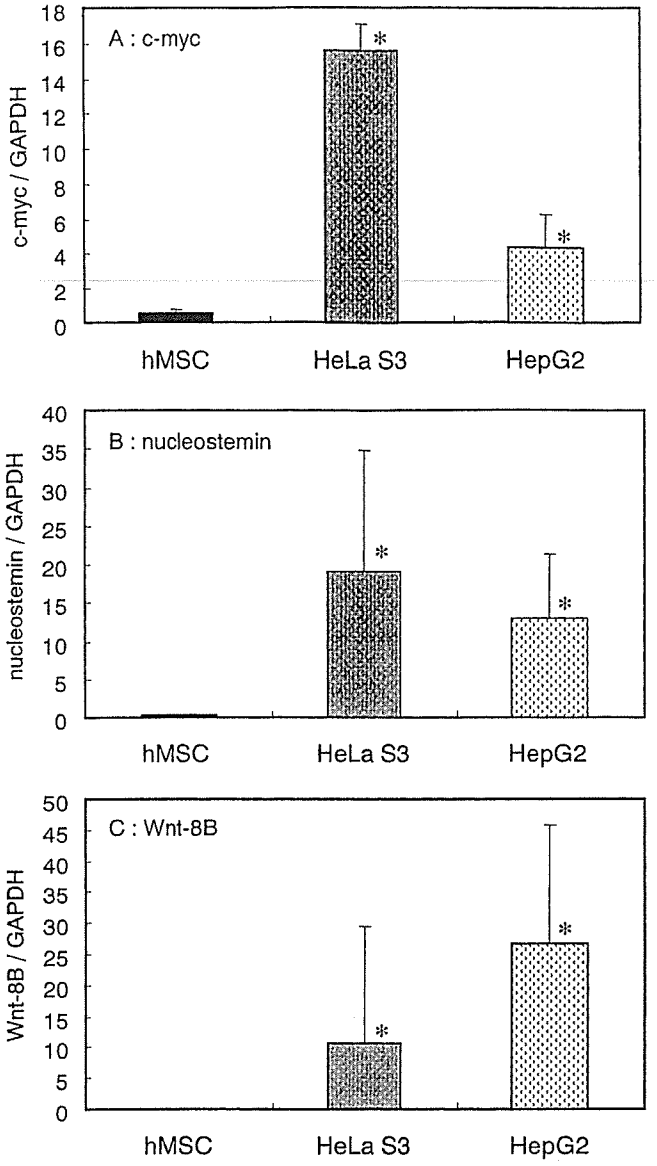


Fig. 6. mRNA expressions of c-myc (**A**), nucleostemin (**B**), and Wnt-8B (**C**) in hMSC, HeLa S3, and HepG2 cells. The expressions of the three genes relative to GAPDH in confluent cultures of hMSC, HeLa S3, and HepG2 cells were investigated by quantitative RT-PCR. Mean values with standard deviations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

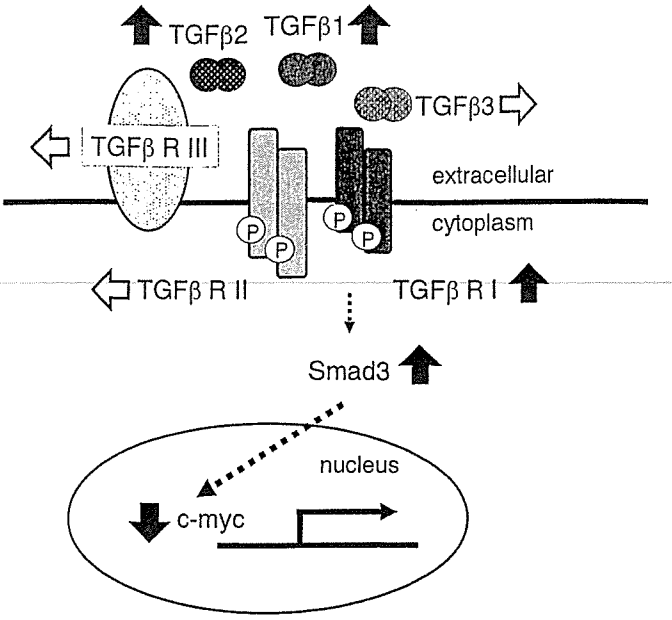


Fig. 7. Changes in the expressions of TGFβ signaling genes during hMSC in vitro culture for 3 months. The *dotted arrows* indicate the TGFβ signal pathway. *White arrows*, no changes; *black arrows*, up or down changes

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.

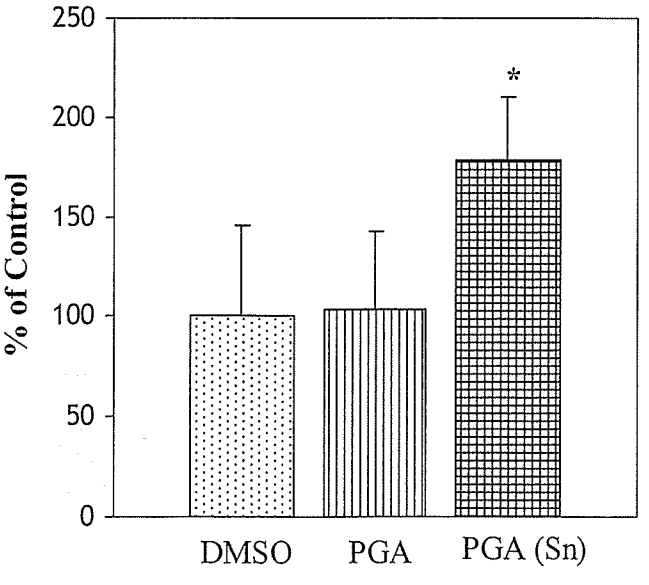

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

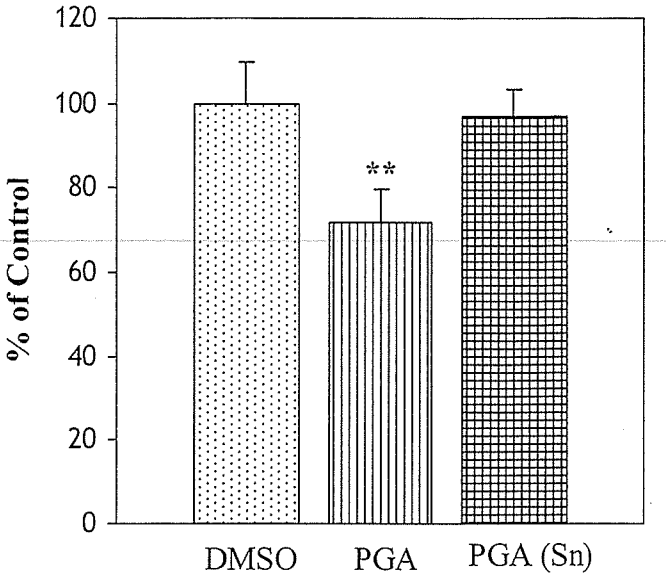

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

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)].

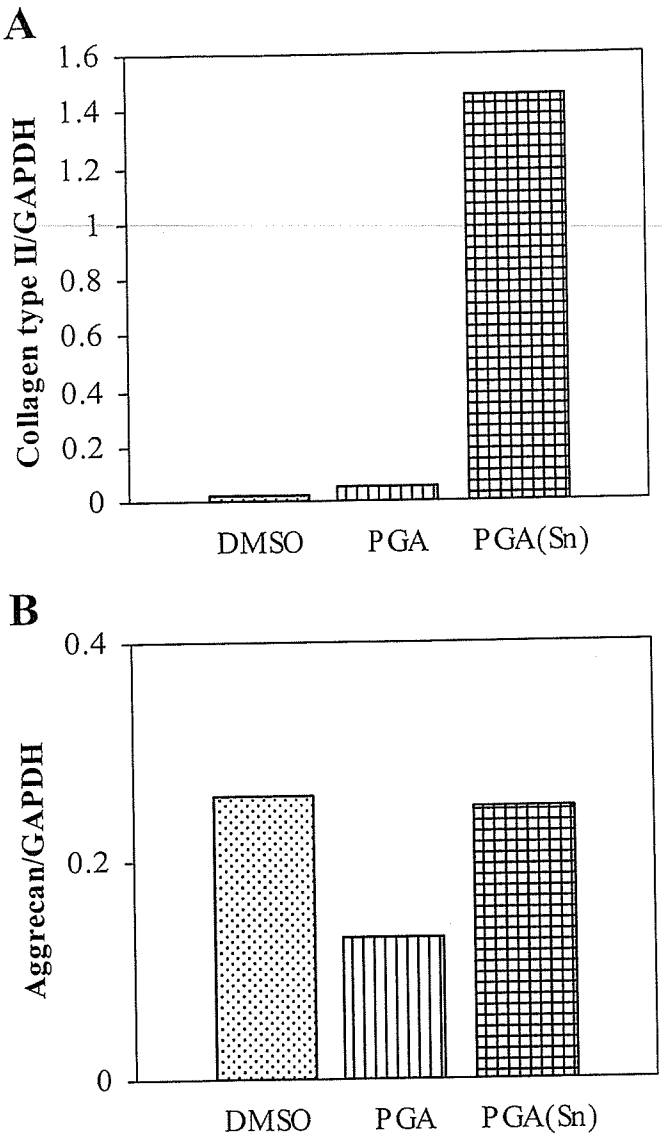

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between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

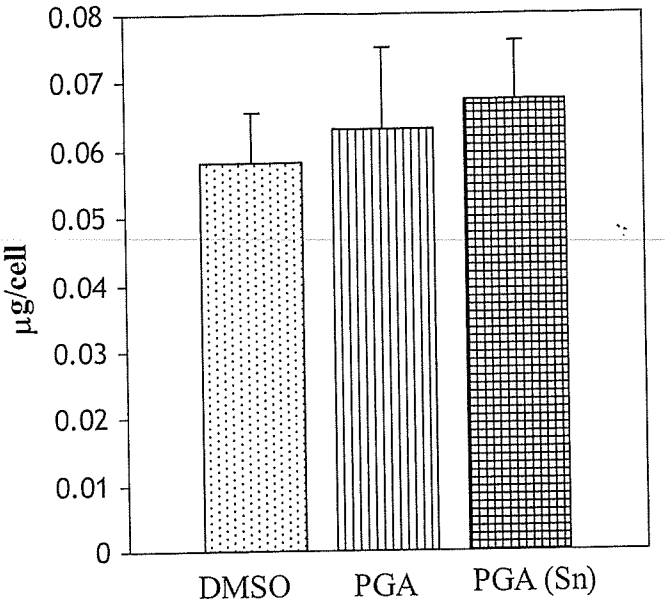

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

(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-

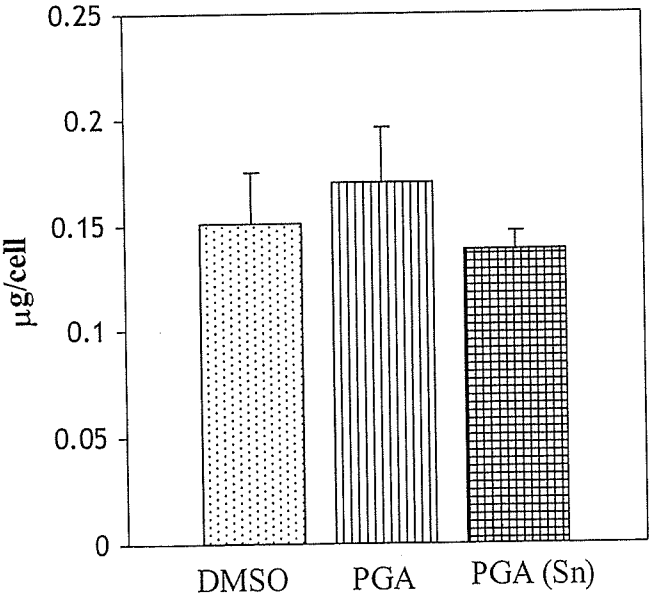

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

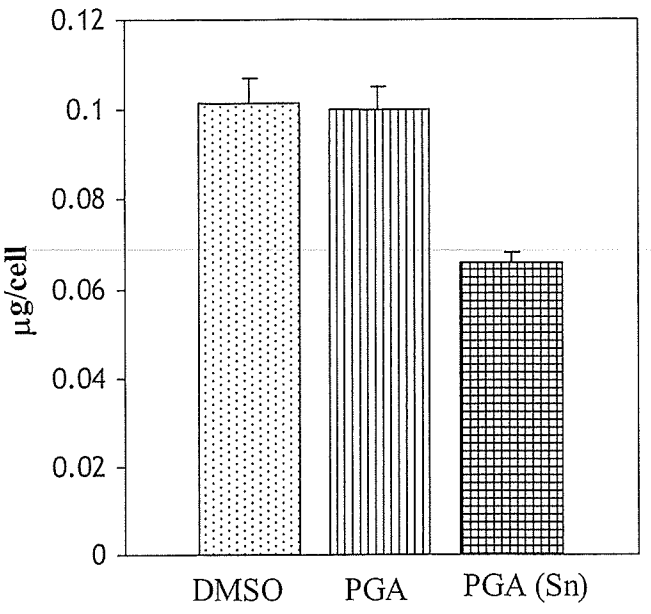

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

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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Effects of overexpression of basic helix–loop–helix transcription factor Dec1 on osteogenic and adipogenic differentiation of mesenchymal stem cells

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Abstract

We recently reported that forced expression of basic helix–loop–helix transcription factor Dec1 accelerated chondrogenic differentiation of mesenchymal stem cells (MSC) in pellet cultures (Shen, M., Yoshida, E., Yan, W., Kawamoto, T., Suardita, K., Koyano, Y., Fujimoto, K., Noshiro, M., Kato, Y., 2002. Basic helix–loop–helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. J. Biol. Chem. 277, 50112–50120). Since MSC have multilineage differentiation potential, we investigated the roles of Dec1 in osteogenic and adipogenic differentiation of human bone marrow-derived MSC. After osteogenic induction of MSC in medium containing dexamethasone, β-glycerophosphate, and ascorbic acid, *Dec1* expression gradually increased from day 5 to day 14, while expression levels of *Dec1* mRNA markedly decreased on days 3 and 7 after adipogenic induction. Infection with adenovirus expressing Dec1 raised mRNA levels of several bone characteristic molecules such as osteopontin, PTH receptor, and alkaline phosphatase, even in the absence of the osteogenic induction medium, although it had little effect on Runx2 expression or calcification. In the osteogenic induction medium, Dec1 overexpression enhanced the expression of osteopontin and alkaline phosphatase and induced matrix calcification. Knockdown of *Dec1* with siRNA suppressed the expression of osteoblastic phenotype by the induced MSC. Using MSC cultures, we also confirmed that forced expression of Dec1 suppressed adipogenic differentiation. These findings suggest that Dec1 modulates osteogenic differentiation of MSC by inducing the expression of several, but not all, bone-related genes.

Keywords: Dec1; Stra13; Bhlhb2; DEC2; Mesenchymal stem cell; Osteogenesis; Adipogenesis

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Introduction

Basic helix–loop–helix transcription factor Dec1 (Stra13/BHLHB2), which was previously identified as a differentially expressed transcript in chondrocytes (Shen et al., 1997), can bind to the CACGTG E-boxes and repress transcription from the target genes (Hamaguchi et al., 2004; Honma et al., 2002; Kawamoto et al., 2004; Li et al., 2003; Sato et al., 2004; St-Pierre et al., 2002; Zawel et al., 2002). Overexpression of Dec1 enhanced chondrogenic differentiation of mouse ATDC5 cells and rabbit bone marrow mesenchymal stem cells (MSC) (Shen et al., 2002), but inhibited adipogenic differentiation of mouse 3T3-L1 cells (Yun et al., 2002); stable expression of Dec2 in mouse C2C12 cells inhibited myogenic differentiation by interacting with MyoD (Azmi et al., 2004). Dec1 also plays a part in circadian rhythm regulation by repressing CLOCK/BMAL1-induced promoters (Butler et al., 2004; Hamaguchi et al., 2004; Honma et al., 2002; Kawamoto et al., 2004; Li et al., 2004; Noshiro et al., 2004; Sato et al., 2004). During endochondral bone development in mouse tibia, *Dec1* mRNA expression was observed from E15.5 in post-mitotic hypertrophic chondrocytes, co-localizing with collagen X mRNA (Maclean and Kronenberg, 2004). At E15.5–E18.5, *Dec1* was also expressed in the primary spongiosa, where Dec1 may be involved in primary bone formation.

Since *Dec1* is expressed in various tissues and induced by various growth factors or hypoxia (Boudjelal et al., 1997; Fujimoto et al., 2001; Miyazaki et al., 2002; Rossner et al., 1997; Shen et al., 1997, 2001), it may be involved in the control of differentiation in numerous tissues. Since MSC can give rise to osteoblasts, chondrocytes, myocytes, and adipocytes (Muraglia et al., 2000; Pittenger et al., 1999), these cells may be useful for analysis of lineage determination and the differentiation steps (Matsubara et al., 2004; Pittenger et al., 1999; Tsutsumi et al., 2001). Among various transcription factors involved in osteogenic differentiation, Runx2 plays a central role in bone formation: In Runx2 null mice, osteoblast differentiation was arrested in both the endochondral and intramembranous skeleton (Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). For adipogenic differentiation, the nuclear hormone receptor PPARγ plays a crucial role in the function of many fat cell-specific genes (Rosen and Spiegelman, 2001). However, numerous other regulatory molecules are likely to be involved in the complex processes of osteogenic and adipogenic differentiation, stage-dependently. To clarify the roles of Dec1 in differentiation of mesenchymal cells, we examined the effects of Dec1 overexpression on osteogenic and adipogenic differentiation of bone marrow MSC: Forced expression of Dec1 up-regulated bone-related gene expression and enhanced calcification, and it suppressed adipogenic differentiation of MSC.

Materials and methods

Cell culture and adenovirus infection

Human MSC were purchased from BioWhittaker (Walkersville, MD) and cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2 atmosphere. For osteogenic induction, MSC were plated at a density of 1×10^4 cells/cm^2 in 16-mm wells coated with type I collagen. For adipogenic induction, MSC were plated at a density of 2×10^4 cells/cm^2 in 16-mm wells.

Twenty-four hours after seeding the cells, infection of these cells with adenovirus expressing human Dec1 (ad-Dec1) or adenovirus expressing LacZ (ad-LacZ) was performed at a multiplicity of infection (MOI) of 50 or 100 pfu/cell, as described previously (Shen et al., 2002). Adenovirus carrying the LacZ gene was generously supplied by Dr. Kohei Miyazono (The University of Tokyo) (Fujii et al., 1999). Six hours later, the culture medium was replaced with an induction medium suitable for osteogenic or adipogenic differentiation.

Osteogenic induction

Thirty hours after plating, MSC were transferred to the osteogenic induction medium (Os-medium), an α-minimal essential medium (αMEM) supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate (Pittenger et al., 1999). As a control, MSC were incubated in αMEM supplemented with 10% FBS alone (medium-A).

Adipogenic induction

For adipogenic differentiation, MSC were transferred to DMEM supplemented with 10% FBS, 0.1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methyl-xanthine (the adipogenic induction medium) (Pittenger et al., 1999). After 3 days, this medium was replaced with DMEM supplemented with 10% FBS and 10 μg/ml insulin (the adipogenic maintenance medium), and the cells were cultured for 24 h in the new medium. Then, the induction/maintenance cycle for adipogenic induction was repeated.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and

Table 1. Sequence of TaqMan probes and primers used for quantitative real-time RT-PCR analysis

Dec1	
TaqMan probe:	5′-CAAGAGTCCGAAGAACCCCCCACAAAA-3′
Forward primer:	5′-GAAAGGATCGGCGCAATTAA-3′
Reverse primer:	5′-CATCATCCGAAAGCTGCATC-3′
ALPase	
TaqMan probe:	5′-CCCCATGCTGAGTGACACAGACAAGAA-3′
Forward primer:	5′-CCGTGGCAACTCTATCTTTGG-3′
Reverse primer:	5′-GCCATACAGGATGGCAGTGA-3′
Osteopontin	
TaqMan probe:	5′-CCTCCTAGGCATCACCTGTGCCATACC-3′
Forward primer:	5′-ATGAGAATTGCAGTGATTTGCTTTT-3′
Reverse primer:	5′-AGAACTTCCAGAATCAGCCTGTTT-3′
PPARγ	
TaqMan probe:	5′-TCAGGGCTGCCAGTTTCGCTCC-3′
Forward primer:	5′-GGTGGCCATCCGCATCT-3′
Reverse primer:	5′-GCTTTTGGCATACTCTGTGATCTC-3′

subjected to real-time quantitative RT-PCR analysis using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) with TaqMan probes and primers shown in Table 1. TaqMan probe and primers for PTH receptor and *GAPDH* were obtained from Applied Biosystems. The values for mRNA levels, relative to internal control *GAPDH*, represent the mean ± SEM for three wells. The experiments were repeated 2–4 times and similar results were obtained each time.

RNA interference

Twenty-four hours after MSC were seeded at a density of 5×10^3 cells/cm^2 in 12-well plates, the cells were transfected with Dec1 siRNA (DHARMACON, CO) or non-silencing control siRNA (Qiagen, CA) using Trans-IT TKO transfection reagent (Mirus, WI). After 48 h incubation, the culture medium was replaced with Os-medium for osteogenic induction.

ALPase staining

Cells were fixed with 3.5% formaldehyde and stained using ALPase staining kit (Muto pure chemical, Tokyo, Japan). The stained cells were rinsed with deionized water and dried overnight.

Alizarin red and von Kossa staining

Fixed cells were incubated with 1% Alizarin red-S (Sigma, St. Louis, MO) for 1 h at room temperature. Alternatively, fixed cells were exposed to sunlight for 30 min in the presence of 5% silver nitrate, and then incubated with 5% sodium thiosulfate for 5 min (von Kossa staining). The stained cells were rinsed with deionized water and dried overnight.

Calcium content

After MSC were rinsed with PBS, calcium in the cell cultures was dissolved in 0.1 M HCl at 4 °C overnight. Calcium content of each sample was determined using a Calcium C kit (Wako pure chemical, Osaka, Japan).

Oil red-O staining

MSC fixed with 3.5% formaldehyde were incubated with 0.3% Oil red-O (Sigma) for 1 h at room temperature. The stained cells were rinsed with deionized water and dried overnight. Lipid drop areas stained with Oil red-O were measured using the NIH image program (RSB, NIMH/NIH, Bethesda, MD).

Results

Changes in *Dec1* mRNA levels during osteogenic differentiation of MSC

Osteogenic differentiation of human MSC was induced by Os-medium. After osteogenic induction, *Dec1* mRNA expression began to increase on day 5 and reached a peak on day 14 (Fig. 1A). In these cultures, mRNA levels of ALPase and PTH receptor started to increase on day 2, reaching a peak on day 14 (Fig. 1B and C); osteopontin mRNA level increased on days 14 and 21 (Fig. 1D).

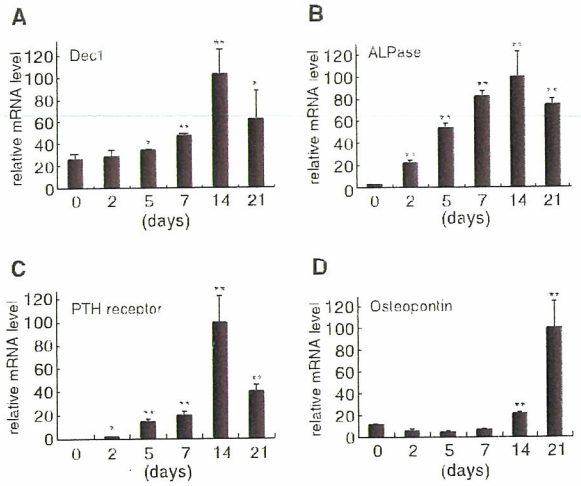


Fig. 1. Changes in mRNA levels of *Dec1*, ALPase, PTH receptor, and osteopontin during osteogenic differentiation of human MSC. Osteogenic differentiation was induced by Os-medium as described in Materials and methods. Total RNA was isolated from the cells on the indicated days after osteogenic induction, and subjected to real-time quantitative RT-PCR analysis. The values represent mRNA levels (mean ± SEM) for *Dec1* (A), ALPase (B), PTH receptor (C), or osteopontin (D) relative to *GAPDH* mRNA levels. *P*-values were calculated by using the Student's *t*-test (***P* < 0.01, **P* < 0.05).

Effect of forced expression of Dec1 and *Dec1* siRNA on osteogenic differentiation of MSC

To test the effect of Dec1 overexpression in the absence of Os-medium, MSC infected with Dec1-expressing adenovirus (ad-Dec1) were maintained in medium-A. Forced expression of Dec1 elevated osteopontin mRNA levels at an MOI 25 or 50 (Fig. 2A) and the increase of osteopontin mRNA levels was significant from day 2 to day 14 compared with infection with LacZ-expressing adenovirus (ad-LacZ) (Fig. 2B). Expression of PTH receptor mRNA was also enhanced from day 5 to day 14, while Runx2 expression was not significantly changed. In addition, the Dec1 overexpression raised the activity and mRNA levels of ALPase (Fig. 2C). However, calcification was not induced by Dec1 overexpression until day 28 (Fig. 2D).

In further studies, the effect of Dec1 overexpression on bone-related gene expression in MSC cultured in Os-medium was examined. Infection of MSC with ad-Dec1 up-regulated osteopontin mRNA levels from day 2 to day 14 in the presence of Os-medium (Fig. 3A). The Dec1 overexpression enhanced the activity and mRNA expression of ALPase (Fig. 3B). The effect of Dec1 overexpression on mRNA levels of osteopontin was much greater than that of Os-medium on day 7, and Dec1 overexpression and Os-medium showed a synergistic or additive effect on day 7 or 14 (Fig. 3C).

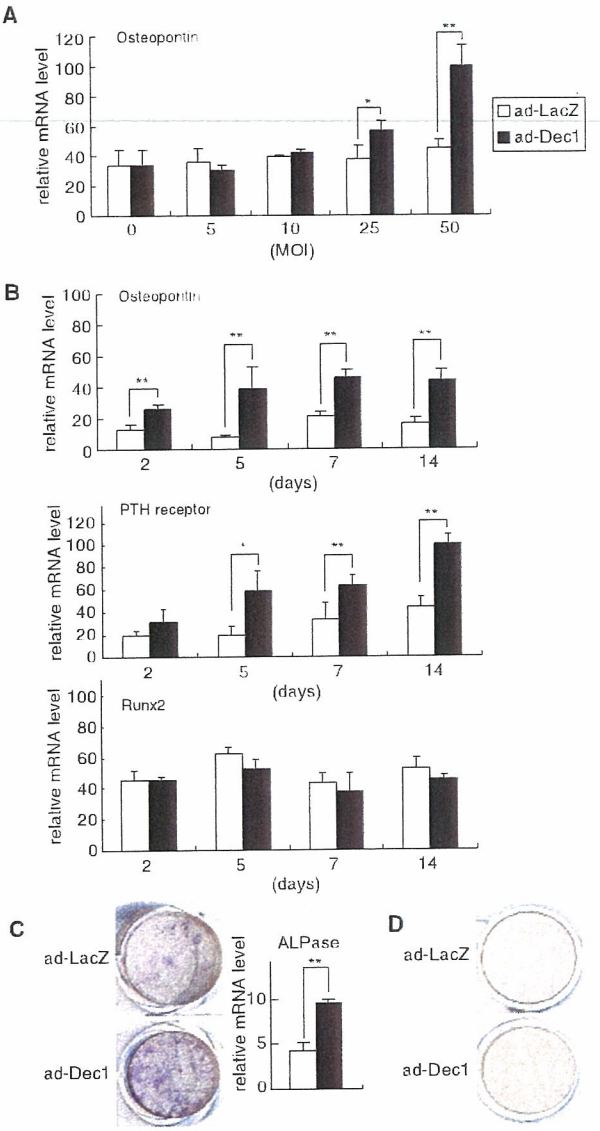


Fig. 2. Effect of Dec1 overexpression on osteopontin, PTH receptor, and ALPase expression in MSC in medium-A in non-osteogenic status. (A) Six hours after MSC were infected with ad-Dec1 at an MOI of 0, 5, 10, 25, or 50, the culture medium was replaced with medium-A. Expression levels (mean ± SEM) of osteopontin mRNA were determined after a 2-day incubation. (B) MSC infected with ad-Dec1 or ad-LacZ at an MOI of 50 were cultured in medium-A; mRNA levels (mean ± SEM) for osteopontin, PTH receptor, and Runx2 were measured on the indicated days. (C) ALPase activity on day 14 was demonstrated by staining the cells with ALPase staining kit. Expression levels of ALPase mRNA (mean ± SEM) were also determined. (D) Matrix calcification on day 28 was examined using von Kossa staining. ***P* < 0.01, **P* < 0.05 (Student's *t*-test).

Furthermore, Dec1 overexpression promoted calcification of MSC cultures in Os-medium: The calcium level in the cultures overexpressing Dec1 on day 25 was 5

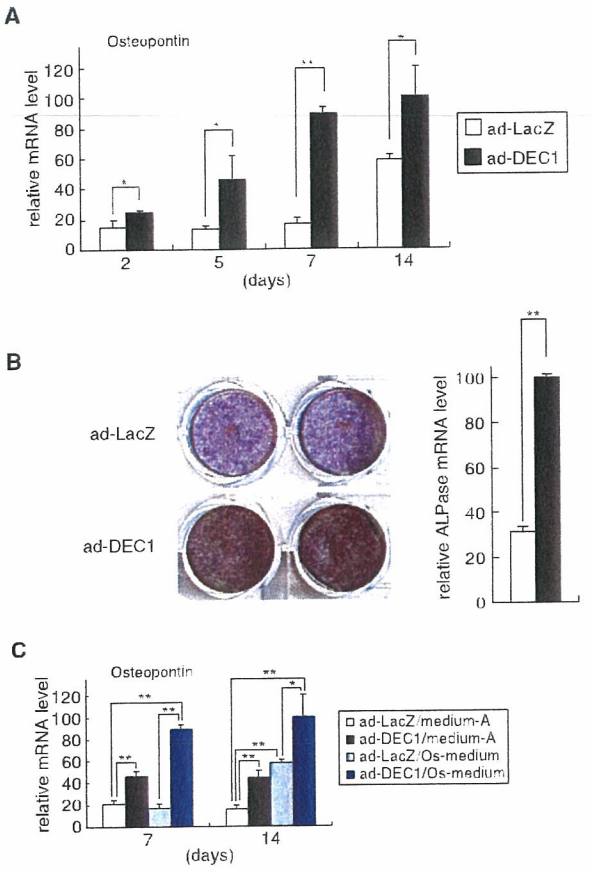


Fig. 3. Effect of Dec1 overexpression on osteopontin and ALPase expression in MSC in Os-medium. Six hours after MSC were infected with ad-Dec1 or control ad-LacZ at an MOI of 50, the culture medium was replaced with Os-medium. (A) Expression levels of osteopontin mRNA (mean ± SEM) on the indicated days were examined by real-time quantitative RT-PCR analysis. (B) ALPase activity on day 21 was examined by staining the cells with ALPase staining kit. Expression levels of ALPase mRNA (mean ± SEM) were also determined. (C) Comparison of osteopontin mRNA levels in Dec1-overexpressing MSC, MSC induced by Os-medium, and Dec1-overexpressing MSC induced by Os-medium. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).

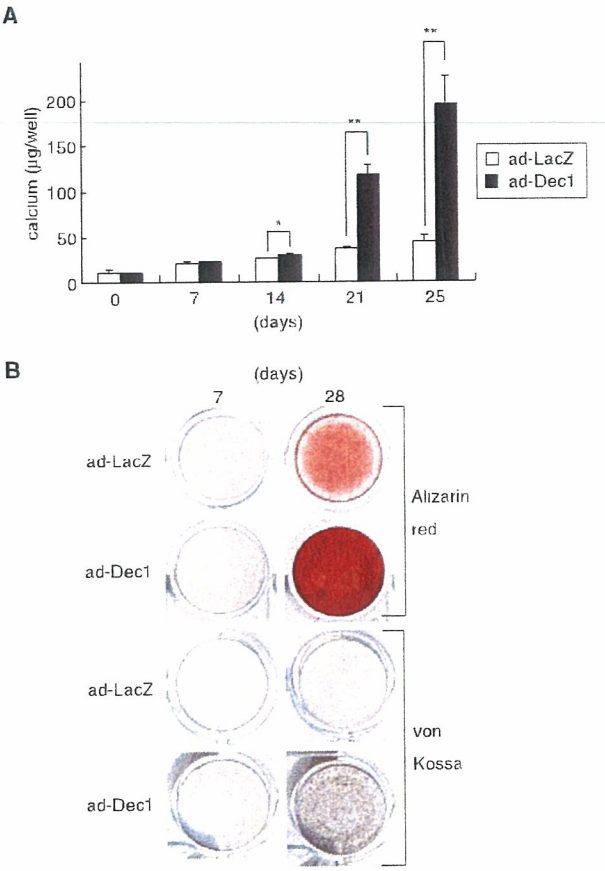


Fig. 4. Effect of Dec1 overexpression on matrix calcification in MSC cultures in Os-medium. (A) MSC infected with ad-Dec1 or ad-LacZ were cultured in Os-medium for the indicated days, calcium content (mean ± SEM) in the cell layers was determined. (B) Matrix calcification in MSC cultures was shown by Alizarin red or von Kossa staining on days 7 and 28. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).

times as high as that in control cultures expressing LacZ (Fig. 4A). The accelerated calcification was confirmed by Alizarin red or von Kossa staining (Fig. 4B). Moreover, PTH receptor expression induced by Os-medium was attenuated by *Dec1* siRNA on days 5 and 7 (Fig. 5), showing the involvement of Dec1 in the osteogenic differentiation process of MSC.

Suppression of adipogenic differentiation of MSC by Dec1 overexpression

To explore the role of Dec1 in adipogenesis, expression levels of *Dec1* during adipogenic differentiation of MSC were examined: After adipogenic induction, mRNA levels for *Dec1* markedly decreased on days 3 and 7 (Fig. 6A), but not on day 14, which suggested that the expression of Dec1 at high levels may suppress adipogenic differentiation. To test this hypothesis, we looked at whether Dec1 overexpression would inhibit adipogenic differentiation of MSC: Infection with ad-Dec1 transiently lowered mRNA levels of PPARγ until day 7 (Fig. 6B), and the Dec1 overexpression also suppressed lipid accumulation on days 3 and 7, but not on day 14 (Fig. 6C), although Dec1 mRNA levels remained high on day 14 (Fig. 6D). These results indicate that Dec1 lowers the rate of adipogenic differentiation only in the early stage.

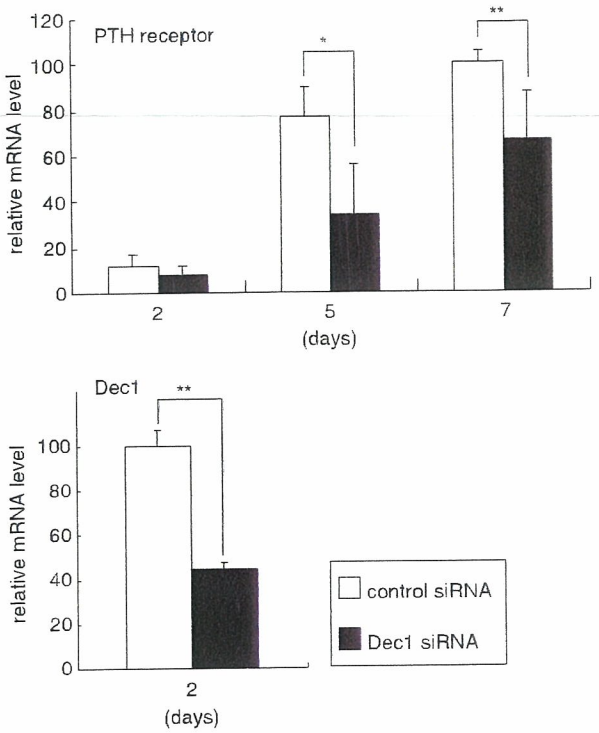


Fig. 5. Effect of RNA interference with *Dec1* in MSC cultures in Os-medium. After MSC were transfected with *Dec1* siRNA or non-silencing control siRNA, the cells were cultured in Os-medium for the indicated days. The values represent mRNA levels (mean±SEM) for *Dec1* and PTH receptor relative to GAPDH mRNA levels. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).

Discussion

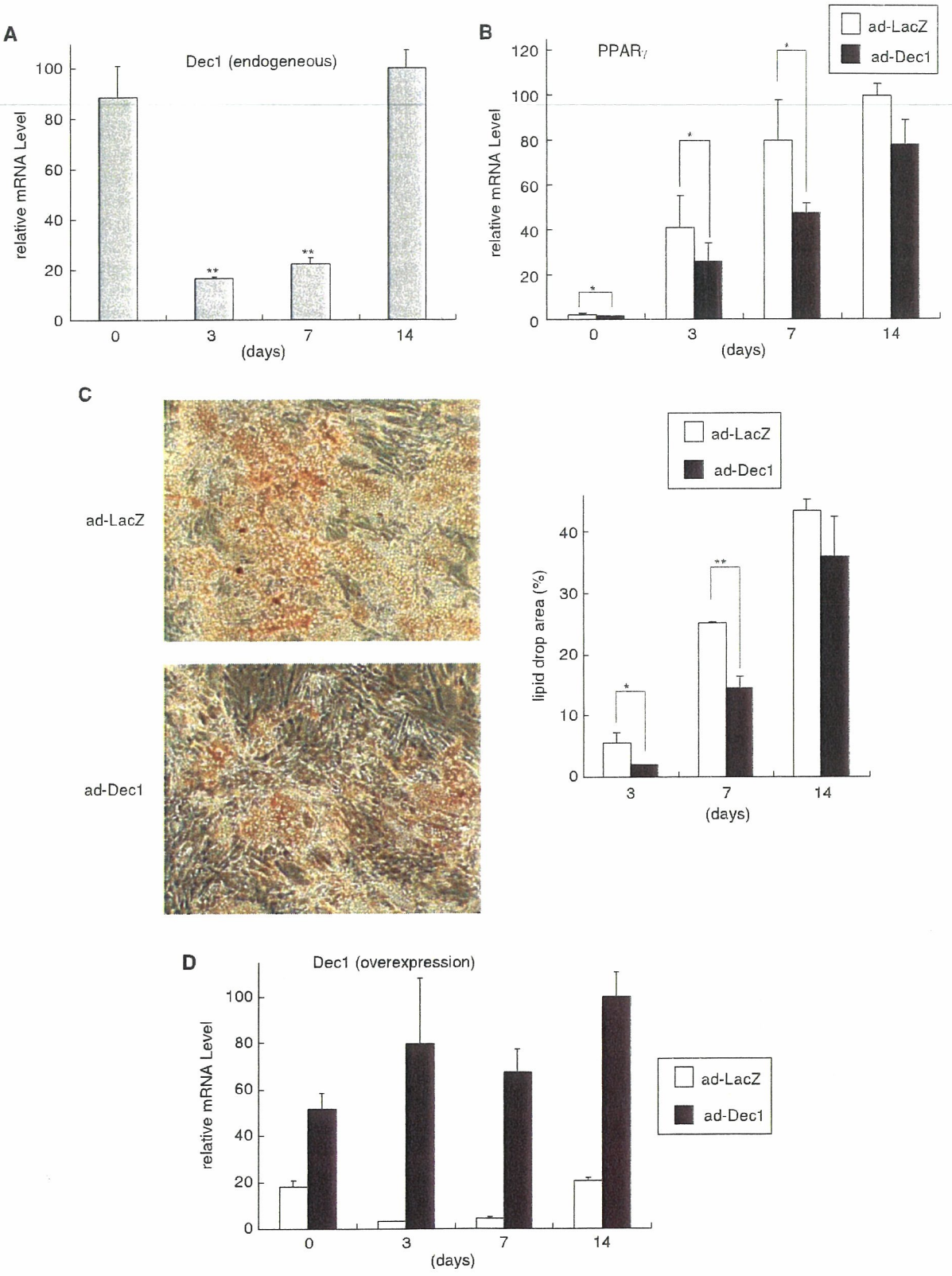
Osteogenic differentiation of MSC is inducible in Os-medium containing dexamethasone, β-glycerophosphate, and ascorbic acid (Matsubara et al., 2004; Pittenger et al., 1999; Tsutsumi et al., 2001): ALPase activity and calcium levels increase a week after osteogenic induction, and the differentiation continues to progress at least until day 21. In the present study, we demonstrated that expression of *Dec1* – as well as bone-related genes – was up-regulated in the induced MSC. Furthermore, forced expression of Dec1 in uninduced MSC up-regulated the expression of some bone-related proteins – such as osteopontin, PTH receptor, and ALPase – and accelerated the osteogenic differentiation and calcification in MSC cultures under osteogenic conditions. Decrease in the *Dec1* mRNA level by about 60% in the presence of siRNA resulted in the suppression of PTH receptor, but it did not decrease the level of osteopontin or ALPase under these culture conditions (data not shown). Furthermore, Dec1 overexpression alone did not induce calcification in MSC cultures. Taken together, these observations suggest that Dec1 is not essential for osteogenesis, but is involved in some aspects of the osteogenic differentiation process.

Since Dec1 overexpression had little effect on expression of Runx2 in MSC, the Dec1-induced osteogenesis could not be attributed to the induction of Runx2. In fact, in vivo studies using Runx2-transgenic mice showed that overexpression of Runx2 in osteoblasts inhibited their maturation (Liu et al., 2001), while Runx2 is essential for osteogenic differentiation in the early stage. These findings suggest that Runx2 stimulates or inhibits osteogenic differentiation of MSC stage-dependently. Some other transcriptional regulators must therefore be involved in the differentiation process of MSC: Dec1 could be one of these transcription factors, although Dec1 alone cannot induce the whole osteogenic differentiation program.

A previous study reported that Dec1 functions as an effector for hypoxia-mediated inhibition of adipogenesis via PPARγ suppression: Stable expression of Dec1 resulted in nearly complete inhibition of adipocyte differentiation of a mouse adipogenic cell line – 3T3-L1 cells (Yun et al., 2002). In the present study, we found that expression levels of *Dec1* decreased only in the initial stage of adipogenic differentiation of MSC, and that Dec1 overexpression suppressed PPARγ expression only in the initial stage. This result, obtained with the primary MSC, revealed the stage-dependent suppression of adipogenesis by Dec1.

Since Dec1 stimulates both osteogenesis and chondrogenesis (Shen et al., 2002), it is not involved in lineage determination, but Dec1 may increase or decrease the rate of differentiation when triggered by other transcription factors: Once the lineage is determined, increased Dec1 possibly enhances the differentiation of MSC into osteoblasts or chondrocytes while

Fig. 6. Effect of Dec1 overexpression on adipogenic differentiation of MSC. MSC were cultured in the adipogenic induction/maintenance medium as described in Materials and methods. (A) Total RNA was extracted from the cells on the indicated days and subjected to real-time quantitative RT-PCR analysis to determine the endogenous *Dec1* mRNA level. (B) Before adipogenic differentiation was induced, MSC were infected with ad-Dec1 or ad-LacZ at an MOI of 100. Relative mRNA levels (mean±SEM) for PPARγ on the indicated days were determined. (C) Lipid accumulation was analyzed using Oil red-O staining. Representative data on day 7 are shown. Percent (mean±SEM) of lipid drop areas stained with Oil red-O were also determined. (D) To confirm overexpression of Dec1 by ad-Dec1 infection, *Dec1* mRNA levels (mean±SEM) in MSC infected with ad-Dec1 or ad-LacZ were determined after the cells were cultured in the adipogenic medium for the indicated days. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).


A
Dec1 (endogeneous)
relative mRNA Level
100
80
60
40
20
0
0
3
7
14
(days)
**
**
B
PPARγ
ad-LacZ
ad-Dec1
relative mRNA Level
120
100
80
60
40
20
0
0
3
7
14
(days)
*
*
*
C
ad-LacZ
ad-Dec1
ad-LacZ
ad-Dec1
lipid drop area (%)
40
30
20
10
0
3
7
14
(days)
*
**
D
Dec1 (overexpression)
relative mRNA Level
100
80
60
40
20
0
0
3
7
14
(days)
ad-LacZ
ad-Dec1

simultaneously inhibiting their differentiation into adipocytes, while decreased Dec1 may facilitate the onset of adipogenic differentiation in the presence of adipogenic induction factors. Thus, Dec1 may work in co-operation with several transcription factors to regulate the rate of osteogenic, adipogenic, or chondrogenic differentiation.

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Circ J 2006; **70:** 1362–1364

Autologous Bone Marrow Mononuclear Cell Implantation Induces Angiogenesis and Bone Regeneration in a Patient With Compartment Syndrome

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A 28-year-old man developed compartment syndrome in the right lower leg after fracture of the tibia and fibula. Despite fasciotomy, many arteries collapsed and union of the tibial and fibula fractures did not occur. Autologous bone marrow mononuclear cell (BMMNC) implantation for therapeutic angiogenesis and subsequent bone regeneration was performed and 4 weeks later, angiography showed a marked increase in collateral vessels surrounding the tibial fracture, and union was completed 6 months later. BMMNC implantation therapy might provide therapeutic angiogenesis and osteogensis in patients with compartment syndrome. (*Circ J* 2006; **70:** 1362–1364)

Key Words: Angiogenesis; Bone marrow mononuclear cells; Bone regeneration; Compartment syndrome

Angiogenesis plays an important role in normal bone development and adult bone healing. Experimental studies have shown that angiogenesis induced by adjunctive modalities, such as angiogenic factors, cytokines, and stem or progenitor cells, contributes to regeneration of bone and repair of fractures[1–3] Recently, it has been shown that autologous bone marrow mononuclear cell (BMMNC) implantation increases collateral vessel formation in ischemic limb models and in patients with limb ischemia[4–6] We present a case of intractable tibia fracture followed by compartment syndrome. In this case, autologous BMMNC implantation promoted angiogenesis, leading to bone regeneration.

Case Report

In January, 2004, a 28-year-old man was involved in a traffic accident. On arrival at the hospital, his right tibia and fibula were fractured and acute compartment syndrome had developed rapidly. Fasciotomy was immediately performed to decompress all compartments of his right leg to prevent ischemic damage to muscles, nerves, blood vessels, and bones. Unfortunately, it was not sufficient and many of the arteries collapsed (Fig 1), causing permanent tissue damage. His right toes became gangrenous, requiring partial foot amputation and split-thickness skin grafts were harvested from his left outer thigh to cover the wound. Although an external fixation device was used to stabilize and align the fractured bones, the tibia did not healed and he could not walk on his foot 6 months later (Fig 2A). Angiography showed severe arterial injury and poor collateral vessel formation in the right lower leg (Fig 3A).

There was no option for conventional therapy, and autologous BMMNC implantation for therapeutic angiogenesis and subsequent bone regeneration was therefore performed. Under general anaesthesia, 700 ml of bone marrow was aspirated from the ileum and collected into plastic bags containing heparin. The BMMNC were immediately sorted using a CS3000-plus blood-cell separator (Baxter, Deerfield, USA) and concentrated to a final volume of approximately 50 ml containing 1.1×10^9 BMMNC. The cells were then

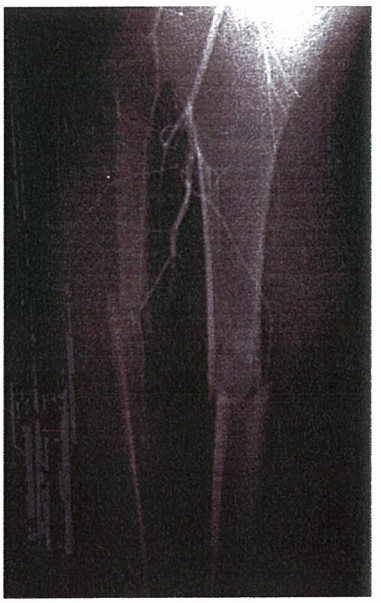

Fig 1. Angiography and radiography show many collapsed arteries and the decreased blood supply below the fractured tibia and fibula, as well as the severe swelling of the gastrocnemius on arrival at hospital.

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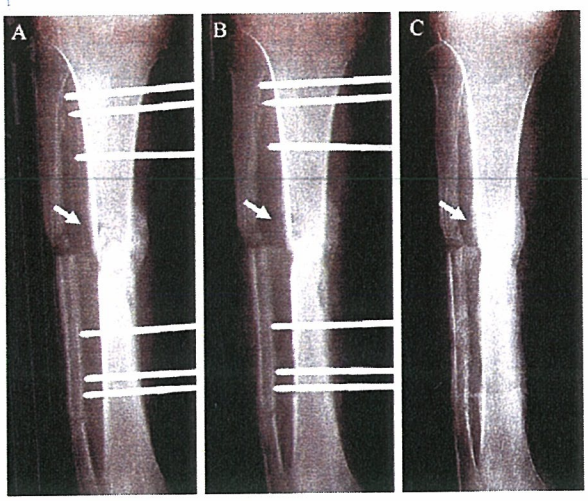

Fig 2. Radiographs of the fractured bones before (Panel A), at 1 month after (Panel B), and 6 months after (Panel C) autologous bone marrow mononuclear cell implantation.

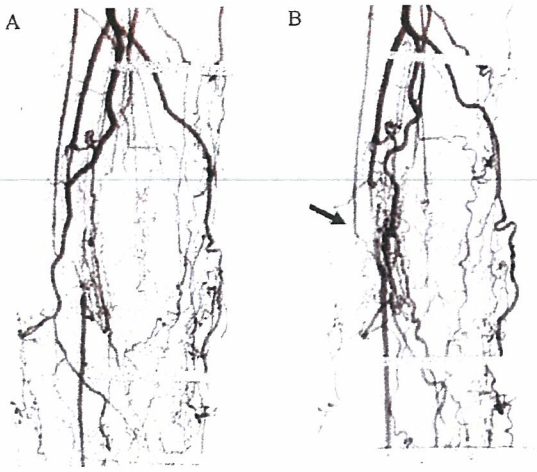

Fig 3. Angiographs of the artery in the injured leg before (Panel A) and at 1 month after (Panel B) autologous bone marrow mononuclear cell implantation.

implanted into the gastrocnemius muscle around the fractured bone as previously described[5,6] Four weeks later, there was marked formation of collateral vessels around the tibial fracture (Fig 3B) and a slight increase in the external callus (Fig 2B). Thereafter, union of the fracture gradually proceeded, and remodeling could be seen. The tibial fracture healed completely and the external fixation device was removed 6 months later (Fig 2C). Furthermore, BMMNC transplantation accelerated wound healing and the open wound on his right foot was completely repaired by partial plastic surgery.

The therapy was approved by the Ethics Committee of the Hiroshima University Graduate School of Biomedical Sciences and the patient gave written informed consent.

Discussion

Fracture of a long bone associated with crushing of structural muscles can often trigger acute compartment syndrome. Although fasciotomy is a useful treatment, delay or insufficient treatment leads to irreversible injury to the muscles, nerves, blood vessels, and bones. There is no option for conventional therapy in cases of poor blood supply in an injured leg. Restoration of bioactivity in the fractured site is thought to be essential for the treatment of a non-union bone fracture. Recently, the effects of various modalities, such as angiogenic factors, cytokines, and stem or progenitor cells, on osteogenesis in animal models have been investigated[1-3]

In the present case, despite immediate fasciotomy, collateral vessel formation remained poor and bone union was impaired in the injured leg. At 6 months after onset, bioactivity of the fractured site was almost lost, and there was little possibility of union of the fracture. Therapeutic angiogenesis by BMMNC implantation has recently been investigated in experimental ischemic limb models and in patients with severe peripheral arterial disease[4-6] It improves limb ischemic symptoms according to angiography in

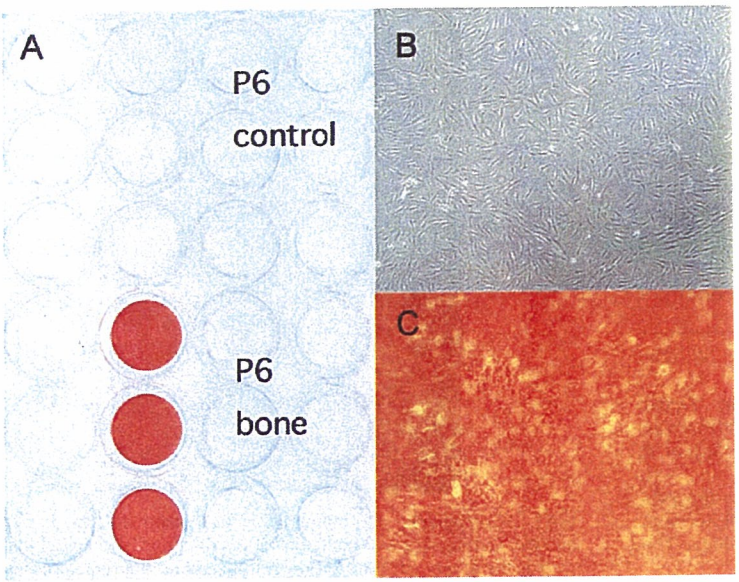

Fig 4. In vitro differentiation of attached cells derived from bone marrow mononuclear cells (BMMNC) into osteocytes. (A) Alizarin red staining after osteogenic differentiation in osteogenic induction medium or control medium. (B) Attached cells derived from BMMNC before osteogenic differentiation. (C) Attached cells after osteogenic differentiation are stained red and form mineralized nodules.