Multi-differentiation Potential of Mesenchymal Stem Cells in Three-dimensional Collagen Gel Cultures

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Introduction

Transplantation from iliac or hip bone has been utilized for grafting bone into defects caused by congenital disease, trauma or tumor invasion, which generally results in a desirable outcome. However, a considerable tissue volume is required for the transplantation, which also necessitates substantial surgical invasion and results in increased morbidity. Additionally, while the restoration of degenerated cartilage in articular condyles has been attempted, this approach has not been successful because of a difficulty in expanding articular chondrocytes *in vitro*. The transplantation of cartilage constructed *in vitro* from expanded primary cells also has had limited success partially because of its inability to sustain function. A newer concept for cartilage regeneration proposes the construction of complex osteochondral tissues consisting of sequential cartilage and bone tissues which may enhance the success of these constructs due to the preferable affinity between bony tissues on transplantation. With these considerations, a new technique for regenerating combined osteochondral tissues *in vitro* has generated substantial research and clinical interest.

An anagenetic technique using mesenchymal stem cells (MSCs) has a good affinity between the organism and material, because MSCs can proliferate quickly and differentiate into various tissues[1]. In combination with a three-dimensional culture system, it seems to be possible to develop a tissue block or scaffold consisting of MSCs, which can alter itself to a tissue component equivalent to peripheral tissue. However, an appropriate scaffold has not yet been developed for a three-dimensional culture system of MSCs.

Of the various three-dimensional scaffold materials, collagen has many advantages over others. Collagen is the major component of extracellular matrix in bone and cartilage, participates in regulating cell growth and differentiation, and because of its mechanical properties and plasticity, provides the relevant physiological stiffness of tissues. Due to its biocompatibility, collagen has been used as a scaffold in constructing a three-dimensional cell culture system, and has demonstrated its ability to maintain a chondrocytic phenotype[2]. Prior to precartilage condensation, the initial stage of chondrogenesis, MSCs highly express type I collagen, and the expressions decrease in a series of chondrocytic differentiations[3,4]. Thus, collagens are essential for maintaining the homeostasis of MSCs, and would possibly be a good candidate as a scaffold for a three-dimensional culture system of MSCs. However, it has not been demonstrated whether or not MSCs can differentiate into osteoblasts or chondrocytes in three-dimensional collagen gels.

The high expression of hyaluronic acid (HA) during endchondral bone formation indicates an important role of HA in bone and cartilage development. In fetal tissues, HA is particularly rich, but the content decreases during development[5]. HA is also a highly and widely distributed component of extracellular matrices, like collagens, in various tissues. HA has been reported to promote the migration and proliferation of MSCs[6,7], and is associated with the acceleration of wound healing[8]. Furthermore, HA binds to specific cell-surface receptors, such as CD44 and the receptor for HA-mediated motility (RHAMM), and also binds to other matrix molecules, such as collagen and proteoglycans[5,9-11]. It would thus be assumed that HA exerts certain influences on the differentiation process from MSCs and becomes an appropriate scaffold for the three-dimensional culture system of MSCs.

The purpose of this study was to characterize the ability of MSCs to differentiate into osteoblastic or chondroctyic lineages in three-dimensional collagen gels under appropriate biological stimuli, and to

elucidate the availability of HA as an additional scaffold for the three-dimensional collagen gel culture system of MSCs.

Materials and Methods

Three-dimensional gel culture for MSCs: Human bone marrow MSCs and growth medium (MSCGM) were purchased from Bio-Whittaker Inc. For the construction of three-dimensional culture, MSCs were harvested from monolayer culture with trypsin treatment and embedded in 0.3% type I collagen gel obtained as a collagen gel kit according to the manufacture's instructions. Briefly, 1 ml collagen solution containing cells either in low density (5 x 10^5 cell/ml) or high density (5 x 10^6 cell/ml) was placed in 24-well cell culture dishes and incubated at 37°C for 30 minutes for gelatinization. For the investigation of HA effect, MSCs were cultured in HA-collagen hybrid gel composed of 0.15% collagen in combination with 0 or 0.5 mg/ml HA (Suvenyl®). The medium was changed every two days up to a maximum of 20 days of culture. All the cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

Cell differentiation: For chondrogenic differentiation, human MSCs seeded at low or high density in collagen gels were placed in chondrogenic differentiation medium (CDM) consisting of serum-free MSCGM that contained 1 mM sodium pyruvate, 100 mg/ml L-ascorbic acid-2-phosphate (AsAP), 1 x 10^{-7} M dexamethasone (Dex), 1% ITS, 5.33 mg linolate, 1.25 mg/ml bovine serum albumin, 40 mg/ml proline, and 10 ng/ml recombinant human TGF-β3[12, 13]. For osteogenic differentiation, human MSCs seeded at low density were cultured osteoblastic differentiation medium (ODM) consisting of MSCGM supplemented with 100 nM Dex, 10 mM β-glycerophosphate, and 0.05 mM AsAP[14].

Toluidine blue staining in the chondrogenic gel: The gels were fixed with 100% ethanol and stained with 1% toluidine blue. To avoid the staining of exogenous HA, the staining procedure was performed at pH 2.5.

Measurement of sulfated glycosaminoglycan (GAG) contents in the chondrogenic gel: The sulfated GAG contents were quantified using a Blyscan Sulfated Glycosaminoglycan Assay kit. The gel cultures stimulated with CDM were digested with papain solution for 3 hours at 65°C. The total GAG contents were quantified by reading an optical density (OD) at 655 nm on a microplate reader. Standard plots were obtained from chondroitin 4-sulfate, and the absolute GAG contents in the gel cultures were determined according to the standard plots with a reference to the OD values.

Alizarin red and alkaline phosphatase (ALP) stainings: The gels cultured in ODM were fixed with 100% ethanol and stained with 1% alizarin red (pH6.3) as described previously. Alkaline phosphatase (ALP) staining was also performed for the gel cultures using fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets.

Calcium incorporation assay: The gels were digested with 0.2% (v/v) triton-X-100, 0.02% collagenase and 6N HCl. The total amount of calcium was measured using a Calcium-C-kit and a microplate reader at an OD of 570 nm.

ALP activity analysis: The gels were digested with 0.2% (v/v) triton-X-100 and 0.02% collagenase, and incubated with 5 mM *p*-nitrophenyl phosphate in 50 mM glycine, 1 mM $MgCl_2$, pH10.5 at 37°C for 2 hours. The ALP activity was estimated by quantifying the absorbance of *p*-nitrophenol product formed at an OD of 405 nm on a microplate reader.

Quantitative real time reverse transcription polymerase chain-reaction (real time RT-PCR): Quantitative RT-PCR was performed for several bone and cartilage markers including type II collagen, type X collagen, type I collagen, bone sialoprotein (BSP) and ALP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control gene. Total RNA was isolated from the gel cultures using a guanidine thiocyanate method. A single strand cDNA was synthesized from 1 mg of total RNA using Oligo $(dT)_{20}$ primer and a Rever Tra Ace-a first strand cDNA synthesis kit. The mRNA levels were determined by quantitative real time RT-PCR analysis, using a SYBR Green PCR master mix or a TaqMan Universal PCR master mix with an automated fluorometer. Quantification of the signals was performed by normalizing the signals of target genes relative to the GAPDH signals. Normalized Ct values were expressed relative to the controls.

Results

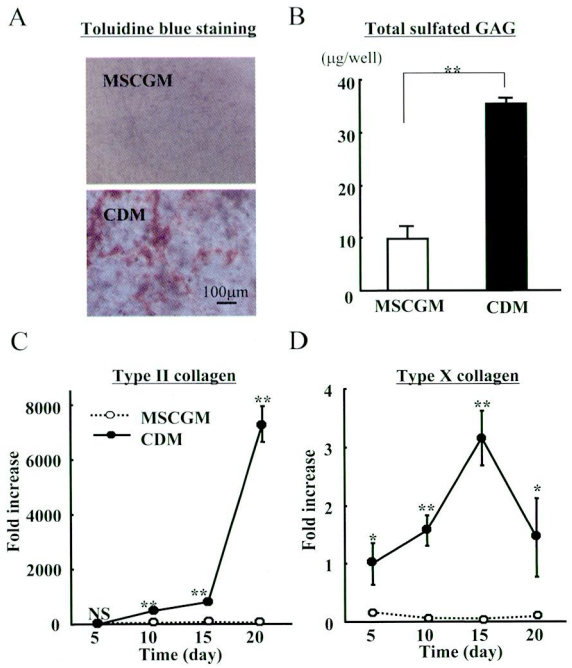

Figure 1 Chondrogenic differentiation in the three-dimensional collagen gel.
MSCs were cultured in three-dimensional collagen gel treated with MSCGM and CDM for 20 days. The gels were fixed and stained with toluidine blue (A). The gels were digested and the GAG contents were measured (B). The mRNA expressions of type II collagen (C) and type X collagen (D) were analyzed with quantitative real-time PCR. *: $p<0.05$, **: $p<0.01$

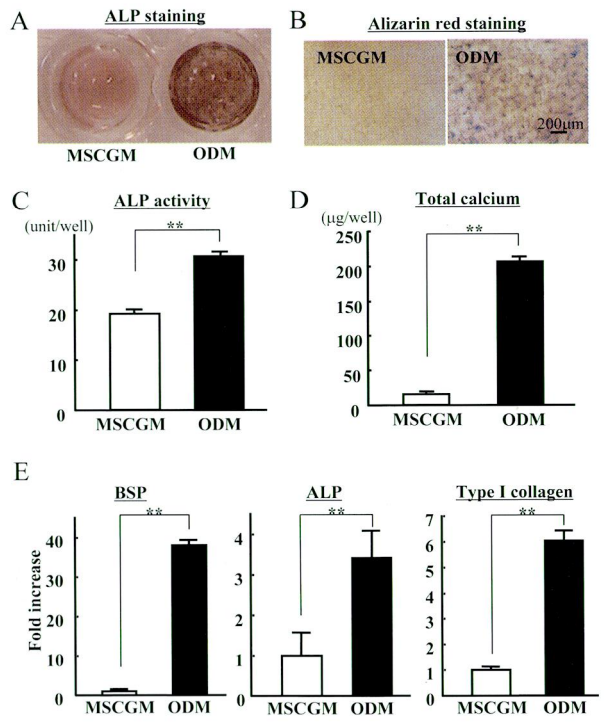

Figure 2 Osteogenic differentiation in the three-dimensional collagen gel.
MSCs were cultured in three-dimensional collagen gel treated with MSCGM and ODM for 14 days. The gels were stained with ALP(A) and Alizarin red (B). The gels were digested, and ALP activity (C) and calcium contents (D) were determined. The mRNA expressions of BSP, ALP and type I collagen in MSCs on day 5 were analyzed with quantitative real-time PCR (E). **: $p<0.01$

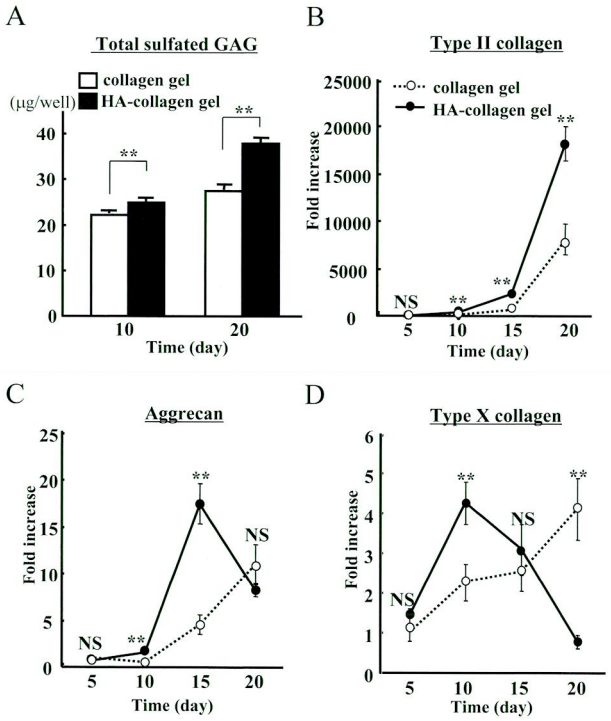

Figure 3 The effect of HA in the chondrogenic differentiation.
MSCs were cultured in HA-collagen hybrid gel treated with MSCGM and CDM for 20 days. The gels were digested and the GAG contents were measured (A). The mRNA expressions of type II collagen (B), Aggrecan (C) and type X collagen (D) were analyzed with quantitative real-time PCR. *: $p<0.05$, **: $p<0.01$

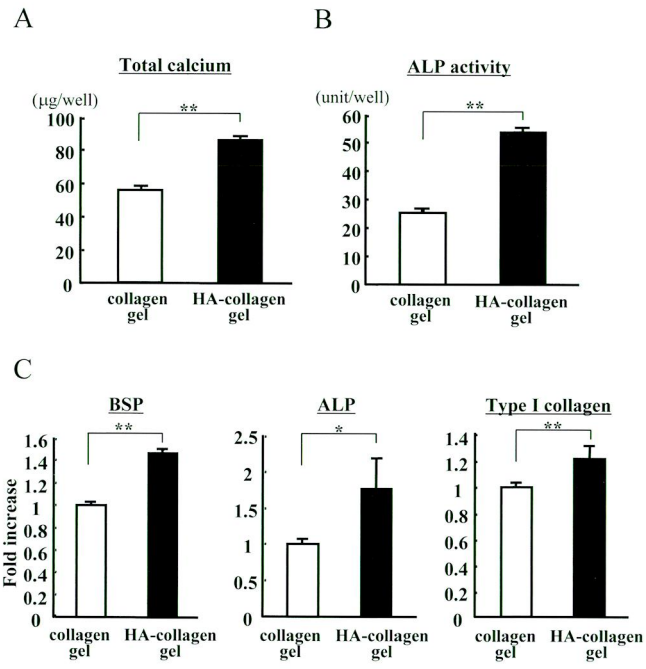

Figure 4 Figure 4 The effect of HA in the osteogenic differentiation
MSCs were cultured in HA-collagen hybrid gel treated with MSCGM and ODM. The gels were digested, and calcium contents (A) and ALP activity (B) were determined on day 14. The mRNA expressions of BSP, ALP and type I collagen in MSCs on day 5 were analyzed with quantitative real-time PCR(C). *: $p<0.05$, **: $p<0.01$

Chondrogenic differentiation in three-dimensional collagen gel culture: The GAG deposition on day 20 assessed by toluidine blue staining was remarkably higher in the high density gel cultures maintained in CDM than in MSCGM as the control (Fig. 1A). Similarly, the total amount of GAG per gel was also significantly higher in constructs maintained in CDM than in MSCGM (Fig. 1B). A time-dependent increase in the expression of type II collagen mRNA was observed in the high density gel cultures treated with CDM compared to MSCGM. This increase was significantly higher in CDM-stimulated gels over control gels at 10, 15 and 20 days of culture, but not at 5 days culture (Fig. 1C). A temporal increase in expression of type X collagen was also observed in the gels treated with CDM, which was significantly greater than that in the control gels at 5, 10, 15 and 20 days of culture (Fig. 1D).

In contrast to the increases in GAG and the expression of type II collagen and type X collagen mRNAs in high density cultures stimulated with CDM, low density gel-cell constructs did not show any significant increases in GAG after 20 days of culture (data not shown). Similarly, the expressions of type II collagen and type X collagen mRNAs were also not changed in the low-density cultures exposed to CDM as compared to those in MSCGM over 15 days (data not shown).

Osteogenic differentiation in three-dimensional collagen gel culture: Intensity of ALP staining was higher in the gel culture maintained in ODM for 14 days than in MSCGM as the control (Fig. 2A).

Alizarin red staining for the MSC gel culture showed enhanced calcium deposition in the extracellular regions of gels maintained in ODM over those in MSCGM (Fig. 2B). The ALP activity and incorporation of calcium into the extracellular matrix were also significantly higher in the gels maintained in ODM than in MSCGM (Fig. 2C, D). The expressions of bone markers BSP, ALP and type I collagen mRNAs were significantly higher in the MSC gel cultures maintained in ODM than those in MSCGM over a period of 5 days (Fig. 2E).

Chondrogenic differentiation in the HA-collagen hybrid gel: The total amount of sulfated GAG in the collagen gels was also significantly greater in gels containing HA than those without it (Fig. 3A). A time-dependent increase in the expression of type II collagen mRNA was observed in both collagen and HA-collagen gels; however, the expressions of type II collagen mRNA in the HA-collagen hybrid gels were substantially greater than those in the collagen mono gels on day 20 (Fig. 3B). A transient up-regulation of the aggrecan and type X collagen was observed in the MSCs cultured in the HA-collagen hybrid gels when treated with CDM, whereas the expression of these genes was increased in a time-dependent manner in those cultured in collagen mono gels (Fig. 3C, D).

Osteogenic differentiation in the HA-collagen hybrid gel: When the MSCs were stimulated by ODM, the incorporation of calcium into the extracellular matrix and ALP activity were significantly greater in the HA-collagen hybrid gel than in the collagen mono gel (Fig. 4A, B).

The gene expressions of all bone markers examined, BSP, ALP and type I collagen were significantly greater in the HA-collagen hybrid gel than in the collagen mono gel (Fig. 4C).

Conclusions

We have demonstrated that human MSCs have an ability to differentiate into both bone and cartilage tissues in three-dimensional collagen gel, and had a higher differentiation potential to hard tissues such as the bone and cartilage in the HA-collagen hybrid gel than in the collagen mono gel. This report indicates that HA treatment has become a useful tool for a more efficient and optimal hard tissue regeneration from MSCs.

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