Differentiation of human bone marrow mesenchymal stem cells to chondrocytes for construction of three-dimensional cartilage tissue

Chikayoshi Matsuda,[1], Mutsumi Takagi,[1*] Takako Hattori,[2] Shigeyuki Wakitani,[3] and Toshiomi Yoshida[1]

[1]International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan, [2]Department of Orthopedic Surgery, Osaka-Minami National Hospital, 2-1 Kidohigashi-machi, Kawachinagano, Osaka 586-8521, Japan, [3]Orthopedic Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan
* Corresponding author (present address);
Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, 060-8628, Japan.
Tel +81-11-706-6567, Fax +81-11-706-6567
E-mail takagi-m@eng.hokudai.ac.jp

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Abstract

A differentiation method of human bone marrow mesenchymal stem cells (MSCs) to chondrocytes was developed for the construction of a three-dimensional (3D) cartilage tissue. The adhesive cells, which were isolated from a human bone marrow aspirate were embedded in type Ⅰ collagen in a poly-L-lactate-glycolic acid copolymer (PLGA) mesh and cultivated for 4 week together with growth factors. The degree of cellular differentiation was estimated by quantitative RT-PCR of aggrecan and type Ⅱ collagen mRNAs and by staining with Safranin O. The 3D culture showed a higher degree of differentiation even without growth factors than the conventional pellet culture with growth factors, namely, dexamethasone and transforming growth factor (TGF)-β3. The 3D culture for 2 week with the combined addition of dexamethasone, TGF-β3, and insulin-like growth factor (IGF)-Ⅰ reached a 30% expression of aggrecan mRNA compared with that in primary human chondrocytes, while the aggrecan mRNA expression in the conventional pellet culture was less than 2%. The sequential two-step differentiation cultivation, during which the cells were cultivated in 3D for 1 week after the conventional two-dimensional (2D) culture for 1 week, could markedly accelerate the expression of aggrecan mRNA compared with the 3D cultivation for 2 week.

Introduction

Mesenchymal stem cells (MSCs) in adult bone marrow have been shown to give rise to multiple mesodermal tissue types, including bone and cartilage (Caplan , 1991), tendon (Caplan et al., 1993), muscle (Wakitani et al., 1995), fat (Dennis and Caplan, 1996), and marrow stromal cells (Majumdar et al., 1995). MSCs have an adherent, fibroblast-like morphology, and have surface antigens characterized as $CD45^-$, which is a representative marker of hematopoietic cells, and $CD105^+$, which is the TGF-β receptor endoglin (Pittenger et al., 1999, Barry et al., 1999). These stem cells are isolated from bone marrow aspirates and present exciting opportunities for cell-based therapeutic applications because of their multilineage potential. In fact, therapeutic modalities have been described for the use of MSCs in cartilage (Wakitani et al., 1994), bone (Bruder et al., 1998), tendon (Young et al., 1998), and bone marrow stroma (Lazarus and Caplan, 1995) regeneration.

The expansion of MSCs after isolation from bone marrow is necessary for their application to regenerative medicine, because the number of MSCs in bone marrow aspirate is very small (e.g., 2,000 cells/ml (Bruder et al., 1997)). It has been reported that human bone marrow mesenchymal stem cells could be passaged in medium containing fetal calf serum (FCS) up to the 38th population doubling level (PDL) while still maintaining their osteogenic potential (Bruder et al., 1997).

The differentiation of MSCs to chondrocytes by the pellet cultivation method employing medium with growth factors, namely, transforming growth factor (TGF)-β3 and dexamethasone, has been reported (Alastair et al., 1998). Other growth factors such as TGF-β1, bone morphogenic protein (BMP)-2, and insulin-like growth factor (IGF)-1 have also been reported to be effective on the differentiation (Allison et al., 2000; Manas et al., 2001; Allison et al., 2001). The degree of differentiation was estimated in these reports by dot-blot analysis of aggrecan and type Ⅱ collagen mRNA expression and by staining with Safranin O and alucian blue. However, there have been few reports on the quantitative estimation of the differentiation of MSCs to chondrocytes.

The size of the pellet (less than 1 mm) is very small compared with the size of the cartilage tissue (more than 5 mm in radius) required for regenerative medicine. That could be the reason why several kinds of three-dimensional (3D) cultivation methods, such as the gel-embedded cultures and porous carrier cultures of primary chondrocytes, have been studied. However, because of gel shrinkage, it is difficult to obtain a uniform cell distribution in porous carriers such as sponge and mesh. We previously developed a novel 3D culture method for chondrocytes to obtain uniform cell distribution and to eliminate the shrinkage by the combination of collagen gel and poly-L-lactate-glycolic acid copolymer (PLGA) mesh (Takagi et al., 2004b). Moreover, no report on the differentiation of MSCs to chondrocytes in 3D culture has been published, except for that employing gelatin sponge (Ponticiello et al., 2000).

For the construction of 3D cartilage tissue, the differentiation of MSCs to chondrocytes and 3D tissue formation were considered as a one-set process, and the optimization of differentiation degree was investigated quantitatively in this study.

Materials and methods

Cell preparation and culture methods

Bone marrow aspirate obtained by routine iliac crest aspiration from human donors (age: 65-73) was diluted with growth medium, plated in a dish (55 cm^2, Corning, Tokyo) to a concentration of 6.0×10^5 nucleated cells/cm^2 and cultured at 37℃ in a humidified atmosphere containing 5% CO_2, during which the medium was changed on days 1, 2, 9 and 16. On day 19, the cells were detached using trypsin-EDTA (Sigma, St. Louis, MO,

USA), counted by the trypan blue dye-exclusion method and subcultivated at an initial cell density of 1×10^4 cells/cm^2. When the culture reached near confluence, the cells were harvested and employed for further differentiation culture. The content of $CD105^+$ $CD45^-$ cells among the cells analyzed by flowcytometer was approximately 90% (data not shown). (Takagi et al., 2004a)

Media

The growth medium was DMEM-LG (Gibco, NY, USA) supplemented with 10% FCS (Gibco), 2500 U/l penicillin, and 2.5 mg/l streptomycin.

The basal differentiation medium was DMEM-HG (Gibco) supplemented with 10% FCS, 2500 U/l penicillin, 2.5 mg/l streptomycin, 50 μg/ml l-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka), 100 μg/ml sodium pyruvate (Wako), and 40 μg/ml proline (Wako). Growth factors, namely, 10 ng/ml transforming growth factor-β3 (TGF-β3, Peprotech), 39 ng/ml dexamethasone (ICN Biomedicals), and 100 ng/ml insulin-like growth factor-I (IGF-I, Peprotech), were added.

The gelling medium consisted of 30.5 g/l DMEM-HG, 35.7% FCS, 8930 U/l penicillin, 8.93 mg/l streptomycin, and 179 μg/ml l-ascorbic acid 2-phosphate.

Pellet cultivation

High-density pellet cell culture was initiated by centrifugation (500×g for 5 min) of 5×10^5 cells suspended in 0.5 ml of the differentiation medium in 15-ml conical tubes. The pellet was incubated for 2 to 4 week at 37℃ in 5% CO_2, during which the medium was changed weekly.

Two-dimensional (2D) differentiation cultivation

Cells were cultured on a dish (55 cm^2, Corning, Tokyo) at a density of 2.0×10^4 cells/cm^2, which corresponded to full confluence, employing the growth medium and then allowing the cells to attach for 6 h at 37℃ in 5% CO_2. Then, the medium was replaced with the differentiation medium and cells were cultivated for 1 week.

Three-dimensional (3D) differentiation cultivation

Eight pieces of PLGA mesh (6 mm φ, 0.25 mm thickness, Vicryl Mesh 910™, Johnson & Johnson, Tokyo, Japan) were laid in a 96 multi-well plate for suspension culture (Sumiron, Osaka, Japan). The cell suspension (3.57×10^7 cells/ml) in gelling medium was mixed with 2.57 aliquot volume of type 1 collagen (0.5%, pH 3, Kokencellgen I-PC™, Koken, Tokyo, Japan) employing a vortex mixer in ice bath. After the cell mixture in collagen (65 μl) was poured into the 96 multi-well plate and incubated at 37℃ for 30 min, the differentiation medium (260 μl) was added and the gel was incubated for 24 h at 37℃ in 5% CO_2. Then, the gel was transferred into a 24 multi-well plate and then incubated for 4 week at 37℃ in 5% CO_2 together with 1.8 ml of differentiation medium, during which the medium was changed weekly.

Cell number analysis

The pellet was hydrolyzed at 37℃ for 40 min with a mixture of 5 g/l trypsin (Sigma), 5 g/l type II collagenase (Worthington Biochemistry), and 5 g/l type I collagenase (Wako, Osaka, Japan). The collagen gel culture was hydrolyzed at 37℃ for 3 h using 2.5 g/l collagenase. Then, the cell concentration was determined by the trypan blue method.

Staining

The pellet and collagen-gel cultures were rinsed twice with PBS, fixed in 20% formalin,

dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, and embedded in paraffin. Sections of 3 μm thickness were cut through the center of the pellets and gel, and were stained with 1% Safranin O in 1% sodium borate.

RNA preparation and RT-PCR

Total RNA was extracted from cells before and after the pellet and the gel-cultures (n=3) using the RNeasy mini Kit (Qiagen, Australia, Victoria). Dnase-treated RNA was used to produce cDNA using Omniscript and Sensiscript RT Kits (Qiagen) and the Gene Amp PCR System 9700 (Applied Biosystems, USA, Foster City). PCR was performed with the cDNA using a HotStar Tag Master Mix Kit (Qiagen) and ABI PRISM 7700 (Applied Biosystems) using actin as the standard (NM 001101, Applied Biosystems). The sequences of primers and probes are listed in Table 1. The cDNA prepared with RNA isolated from primary human chondrocytes from an articular cartilage was also employed as a positive control for the PCR analysis. The ratio of mRNA expression in cultured cells to that in primary chondrocytes was calculated as the degree of expression.

Results

Effect of growth factors on the expression of aggrecan mRNA in 2D culture

Two-dimensional differentiation cultures were performed for 7 days employing differentiation medium supplemented with several combinations of growth factors, and the degree of expression of aggrecan mRNA was determined (Fig. 1). There was almost no change in cell density during culture (data not shown). The degrees of expression in the cultures without the growth factors and with TGF-β3 plus IGF-1 were less than 1.5% and showed almost no increase during culture. On the other hand, the addition of TGF-β3 plus dexamethasone increased the expression during the culture, which reached 6.3% on day 7. Moreover, the combined addition of these three growth factors caused a rapid increase in the degree of expression of aggrecan mRNA during culture, reaching 23% on day 7. Prolonging the differentiation culture in 2D to 2 week resulted in cell detachment and death (data not shown).

Differentiation in 3D culture

Differentiation in 3D and pellet cultures were examined for 4 week. Degrees of expression of aggrecan and type II collagen mRNA in inoculum cells were 0.047 and 0%, respectively. The degrees of expression at 2 week are shown in Fig. 2. While the pellet culture with TGF-β3 plus dexamethasone showed only 0.15% aggrecan mRNA expression, the 3D culture showed 3.3% even without any growth factor. The addition of TGF-β3 and dexamethasone with or without IGF-1 to the 3D culture markedly increased the degree of expression to 17 and 28%, respectively. On the other hand, the degrees of expression of type II collagen mRNA in the pellet and 3D cultures without any growth factor were less than 0.001%. The addition of TGF-β3 and dexamethasone with or without IGF-1 to the 3D culture markedly increased the degree of expression of type II collagen mRNA to 0.01 and 0.03%, respectively.

Sections of cultures at 4 week were stained with Safranin O (Fig. 3). Three-dimensional culture at day 1 showed no staining. The center of the pellet was not stained while the edge part of the pellet was well stained. The staining of the 3D culture at 4 week cultivated with TGF-β3 and dexamethasone with or without IGF-1 was apparently higher than that without any growth factor.

Two-step differentiation culture

In order to accelerate the differentiation, the two-step culture with differentiation medium was studied, in which cells were cultivated for 1 week in 3D after the 2D culture for 1 week, and then compared with the one-step 3D culture (Fig. 4).

The degree of expression of aggrecan mRNA gradually increased during 2 week of the 3D culture. On the other hand, the degree in 2D rapidly increased and reached 23% at 1 week while the degree in the 3D culture was only 8.5% at 1 week. The high degree attained in the 2D culture was well maintained during the 2nd-step 3D culture. Although there was almost no expression of type Ⅱ collagen mRNA at 1 week in both 2D and 3D cultures, the degree increased apparently during 2nd week. The degree of type Ⅱ collagen mRNA expression at 2 week in the 2nd-step 3D culture was markedly higher than that in the one-step 3D culture.

Discussion

The degree of expression of aggrecan mRNA was low and did not increase during the 2D cultures without or with TGF β 3 and IGF-1 (Fig. 1). On the other hand, the combinations of TGF β 3 and dexamethasone with or without IGF-1 resulted in apparently higher expression of aggrecan mRNA and their degrees increased in 1 week. Because employing these three factors resulted in the highest degree of expression, the combined addition of TGF β 3, dexamethasone, and IGF-1 was the best cocktail to stimulate the differentiation of MSCs to chondrocytes in the 2D culture.

The degrees of expression of aggrecan and type Ⅱ collagen mRNAs in the 3D culture even without any growth factor were markedly higher than those in the pellet culture with TGF β 3 and dexamethasone. (Fig. 2) The combined addition of TGF β 3, dexamethasone, and IGF-1 resulted in the highest expression of both aggrecan and type Ⅱ collagen mRNAs. The intensity of Safranin O staining of sections of 3D cultures corresponded with the degrees of expression (Fig. 3). Consequently, it was proved that this 3D culture method was better than the pellet culture from the viewpoint of not only tissue size but also degree of differentiation. The combination of TGF β 3, dexamethasone, and IGF-1 was also the best cocktail for the 3D differentiation culture.

It has been reported that the expression of type Ⅱ collagen mRNA occurs in the later phase compared with that of aggrecan mRNA during the differentiation of MSCs to chondrocytes. (Frank et al., 2001) This might be true also in our case, because the degrees of expression of type Ⅱ collagen mRNA were markedly lower than those of aggrecan mRNA at 1 (Fig. 4) and 2 week (Figs. 2 and 4) of culture.

The darkness of Safranin O staining of pellet was markedly higher than that of 3D culture even with optimum combination of factors (Fig. 3). This might be partly due to the difference in cell density between pellet (approx. 1×10^8 cells/ml with pellet size (approx. 1 mm $\times$ 1 mm $\times$ 0.5 mm)) and 3D gel (approx. 1×10^7 cells/ml). So, increasing the cell concentration in 3D culture may partly solve this problem.

The nonstained area at the center of the pellet was not observed in the 3D culture. This suggests that cell necrosis due to the limitation of mass transfer occurred not in the 3D culture but in the pellet culture. (Fig. 3) This may be one of the reasons why the 3D culture even without any growth factor showed a higher degree of expression than the pellet culture with TGF- β 3 and dexamethasone. (Fig. 2) Thus, it was considered that the 2D culture may be better than the 3D culture from the viewpoint of differentiation speed because the mass transfer rate may be higher in the 2D culture than in the 3D culture. This was supported by the finding that the degree of expression of aggrecan mRNA even at 1 week in the 2D culture supplemented with TGF β 3, dexamethasone,

and IGF-1 (23%, Fig. 1) was almost the same as the degree at 2 week in the 3D culture (28%, Fig. 2).
Therefore, the two-step differentiation culture containing 2D and 3D cultures was experimentally compared with the single-step 3D differentiation culture. (Fig. 4) Aggrecan expression level at 1 week in 2D culture (Fig. 4) was comparable to that at 1 week in 2D in Figure 1. On the other hand, the expression level not only at 1 week but also at 2 week in 3D culture in Figure 4 was apparently lower than the level at 2 week in 3D culture in Figure 2. The difference in culture duration may be one of the reasons for this difference in expression level. However, there may be unknown operational factors affecting the activity of cells employed in each culture.

In Figure 4, the degree of aggrecan mRNA expression in the 2D culture increased more rapidly within 1 week compared with that in the 3D culture, and the high level of expression attained in the 2D culture was maintained during the following 3D culture for 1 week. The degree of aggrecan mRNA expression at 2 week in the single-step 3D culture was apparently lower than that at 1 week in the 2D culture. Consequently, the time required to reach a high degree of aggrecan mRNA expression such as 20% was successfully reduced by more than 1 week using the two-step differentiation culture procedure. The 2D and 3D cultures in the two-step culture were mainly for differentiation and accumulation of aggrecan, respectively.

Conclusion

The MSCs from the bone marrow aspirate could be differentiated to chondrocytes in 3D culture containing collagen gel and PLGA mesh by employing the growth factors TGF β 3, dexamethasone, and IGF-1. The two-step differentiation culture using 2D culture followed by 3D culture could successfully reduce the time required for the differentiation.

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Table 1. Sequences used in PCR

Human aggrecan	
Sense	5' - AGTCCTCAAGCCTCCTGTACTCA - 3'
Antisense	5' - GCAGTTGATTCTGATTCACGTTTC - 3'
Probe	5' - ATGCTTCCATCCCAGCTTCTCCGG - 3'
Human type Ⅱ collagen	
Sense	5' - CGCTGTCCTTCGGTGTCAG - 3'
Antisense	5' - CCTTGATGTCTCCAGGTTCTCC - 3'
Probe	5' - CCAGGATGTCCGGCAACCAGGA - 3'

Figure 1. Effect of growth factors on the expression of aggrecan mRNA in 2D culture.
MSCs were cultivated on a dish for 1 week together with the growth factors TGF β 3 (T), dexamethasone (D), and IGF- I (I), and the degree of expression of aggrecan mRNA was determined at day 1 (closed bar), 4 (gray bar), and 7 (open bar). Bar indicates the standard deviation.

Figure 2. Effect of growth factors on differentiation in 3D culture.
Pellet and 3D cultures of MSCs were performed for 2 week employing the growth factors TGF β 3 (T), dexamethasone (D), and IGF- I (I), and the degrees of expression of aggrecan and type II collagen mRNAs were determined. The degree of expression of inoculum MSCs were 0.047 and 0%, respectively. Bar indicates the standard deviation.

Figure 3. Microscopic observation of sections stained with Safranin O.
Pellet and 3D cultures of MSCs were performed for 4 week employing the growth factors TGF β 3 (T), dexamethasone (D), and IGF- I (I), and the sections were stained with Safranin O.

Figure 4. Two-step differentiation cultivation.
MSCs were cultivated in the single 3D (○) or two-step 2D-3D (●) cultures employing medium supplemented with TGF β 3, dexamethasone, and IGF- I , and the degrees of expression of aggrecan mRNA and type II collagen mRNA were determined. Bar indicates the standard deviation.