Synergistic effect of ascorbic acid and collagen addition on the increase in type 2 collagen accumulation in cartilage-like MSC sheet

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Abstract Aiming to increase the content of type 2 collagen in scaffold-free cartilage-like cell sheets prepared using human bone marrow mesenchymal stem cells, the effect of several kinds of additives in a chondrogenic medium was investigated. Addition of ascorbic acid 2 phosphate (VCP) at a high concentration (250 µg/ml) and type 1 atelocollagen (5 µg/ml) increased the accumulation of type 2 collagen by fourfold and twofold, respectively. On the other hand, an antioxidant, glutathione showed no such effect. The synergistic effect of VCP and type 1 atelocollagen resulted in an eightfold increase in the accumulation level of type 2 collagen. Furthermore, the gene expression level of type 2 collagen increased and that of matrix metalloproteinase-13 (MMP-13) decreased to approximately one-third of the control. The increase in type 2 collagen accumulation in the scaffold-free

cartilage-like cell sheet might be due to not only the enhancement of the synthesis but also the suppression of the degradation of type 2 collagen by MMP-13.

Keywords Mesenchymal stem cell · Cartilage-like cell sheet · Type 2 collagen · Ascorbic acid · Synergistic effect

Introduction

Cartilage tissue is composed of only 10 % chondrocytes and 90 % extracellular matrix (ECM), the main components of which are collagens and proteoglycans (Ibold et al. 2009). ECM plays a role in the maintenance of the mechanical properties of cartilage tissue including

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tensile strength due to collagens and elasticity due to proteoglycans (Temenoff and Mikos 2000). Among many types of collagen, type 2 collagen is the main component of ECM in the cartilage. The characteristics of type 2 collagen are the high hydroxyl level of lysine and the high content of a bound carbohydrate group (Eyre and Muir 1975; Temenoff and Mikos 2000). Therefore, tissue-engineering-based cartilage regeneration should lead to ECM recovery. Among the cell sources for cartilage regeneration, mesenchymal stem cells (MSCs) are an attractive candidate because they can be harvested by a minimally invasive procedure and can differentiate into chondrocytes in the presence of TGF- β or other cytokines (Fukumoto et al. 2003; Indrawattana et al. 2004; Johnstone et al. 1998; Mackay et al. 1998; Pittenger et al. 1999; Worster et al. 2000, 2001).

An artificial three-dimensional (3D) cell scaffold is usually utilized to form a 3D cartilage tissue for implantation. Such a scaffold generally consists of synthetic polymers such as poly (D, L-lactide-co-glycolide) (Fujihara et al. 2009; Mercier et al. 2005; Moran et al. 2003) and biological materials such as collagen and alginate (Hao et al. 2010; Ochi et al. 2002; Scotti et al. 2010; Solchaga et al. 2005). However, there are still several issues associated with the long-term safety of these materials (Newman 1998). To avoid risks of unknown disease factors (Martin et al. 2005), such materials should ideally be excluded completely. In this regard, an alternative scaffold-free 3D tissue system is required.

Several methods of scaffold-free cultivation of MSCs to form a cartilage-like cell sheet using a membrane-separated culture vessel have been developed so far (Elder et al. 2009; Maeda et al. 2011; Murdoch et al. 2007). We previously reported a novel method to prepare scaffold-free cartilage-like cell sheets without shrinkage from human bone marrow MSCs using a cell culture insert (Sato et al. 2013). The cell sheets have indicated higher cartilage-related gene expression levels than the pellet culture (Maeda et al. 2011; Murdoch et al. 2007). However, the level of type 2 collagen accumulation (approximately 10 ng/mg) (Sato et al. 2013) in the cell sheet was markedly lower than that in the native cartilage (approximately 100 μ g/mg). Thus, the level of type 2 collagen accumulation in the MSC sheet should be increased.

Although the addition of some sugar composing glycosaminoglycans (GAGs) such as chondroitin

sulfate, *N*-acetyl-D-galactosamine, D-glucuronic acid, D-galactose, and *N*-acetyl-D-glucosamine has been reported to increase the type 2 collagen content (level of accumulation) in 3D culture of chondrocytes (Kagita et al. 2010), no such increase was observed in the sheet of MSCs.

Ascorbic acid is important for collagen formation through the synthesis of hydroxyproline and hydroxylysine in the collagen molecule (Cardinal and Udenfrie 1974). Indeed, it was reported that ascorbic acid generally promotes collagen synthesis (Murad et al. 1981). In addition, several concentrations of ascorbic acid (0–500 mM) were reported to induce proliferation and differentiation of MSCs. It was indicated that ascorbic acid promoted proliferation and ECM secretion. Moreover, ascorbic acid did not show any influence on cell morphology and any toxicity to MSCs. However, this was the case of differentiation into adipocytes and osteoblasts but not into chondrocytes (Choi et al. 2008). Ascorbic acid is also usually added to chondrogenic medium at 50 μ g/ml for the production of collagen by chondrocytes and MSCs (Mackay et al. 1998; Maeda et al. 2011; Nagai et al. 2008). However, to the best of our knowledge, there has been no report about the addition of ascorbic acid at a concentration higher than 50 μ g/ml to a scaffold-free MSC sheet culture.

Furthermore, ascorbic acid and the binding of ECM components such as collagen and fibronectin to the integrin receptor stimulates extracellular signal-regulated kinase (ERK) signaling (Illario et al. 2003; Temu et al. 2010; Ulrich-Merzenich et al. 2007). It was also reported that collagen-coated wells promoted chondrogenic redifferentiation of chondrocytes (Rutgers et al. 2013). Thus, it was supposed that collagen supplemented to the medium may increase the content (accumulation level) of type 2 collagen in MSC sheets.

On the other hand, matrix metalloproteinases (MMPs) are enzymes degrading ECMs components, such as collagen, elastin, and proteoglycans (Ra and Parks 2007). Especially, MMP-13 has significant activity to degrade type 2 collagen, and it cleaves type 2 collagen at least 10 times faster than MMP-1 (Mitchell et al. 1996). MMP-13 gene expression in chondrocytes was reported (Borden et al. 1996) and the increase in its level resulted in the degradation of cartilage ECM (Burrage et al. 2006). Thus, it was supposed that the control of MMP-13 expression is important for the enhancement of type 2 collagen accumulation. However, the effect of ascorbic acid or

type 1 atelocollagen on MMP-13 expression has not been clarified. Consequently, the effects of ascorbic acid at concentrations higher than 50 µg/ml and type 1 atelocollagen on the accumulation of type 2 collagen in scaffold-free cartilage-like MSC sheets were investigated to increase the content (accumulation level) of type 2 collagen in those MSC sheets.

Materials and methods

MSC isolation and cultivation

MSCs were isolated from bone marrow aspirates obtained by routine iliac crest aspiration from human donors (donor A, 54-year-old female; donor B, 19-year-old male). Both donors gave their informed consent in this study, which was approved by our institutional committee on human research, as required by the study protocol. Briefly, bone marrow cells were seeded at a concentration of 6.0×10^5 nucleated cells (NCs)/cm² on a dish (55 cm²; Corning, Corning, NY, USA) with the MSC growth medium containing Dulbecco's modified Eagle's medium–low glucose (DMEM-LG; Life Technologies, Grand Island, NY, USA) supplemented with 10 % FCS (Life Technologies), 100,000 U/l penicillin, and 100 mg/l streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultivated at 37 °C in a humidified atmosphere containing 5 % CO₂, during which the medium was changed with a fresh medium on days 1, 2, and 9, and nonadherent cells were removed. When the cell distribution on a bottom surface of a dish was markedly uniform, the cells were detached using trypsin–EDTA (Sigma) and inoculated onto the original dishes. When the culture reached near confluence, the cells were collected and further sub-cultured at 1.5×10^3 cells/cm². Cells after 3 to 4 passages were used in all experiments.

Preparation of cartilage-like MSC sheets

Cartilage-like MSC sheets were prepared as described previously (Sato et al. 2013). Briefly, MSCs after 3 to 4 passages (18.6×10^5 cells/well) were seeded into cell culture inserts (CCIs) (0.3 cm²; thickness, 20–25 µm; polyethylene terephthalate; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in a 24-well plate containing 1.0 ml of the chondrogenic medium consisting of DMEM-LG containing 1.75 g/l glucose

(Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100,000 U/l penicillin, 100 mg/l streptomycin (Sigma), 50 µg/ml pyruvic acid sodium salt (MP Biomedicals, Solon, OH, USA), 20 µg/ml proline (MP Biomedicals), 50 nM dexamethasone (MP Biomedicals), 0.5 % ITS+ premix (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 µg/ml linoleic acid; BD), 5 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA), 50 ng/ml IGF-1 (PeproTech, Rocky Hill, NJ, USA), and 5 % FCS. All cells were incubated at 37 °C in 5 % CO₂ for 21 days. The medium was replaced with a fresh medium every 2 days. In some cases, 25, 50, 250, or 2500 µg/ml ascorbic acid phosphate magnesium salt (VCP) (Wako), 0.05, 0.5, 5, or 50 µg/ml type 1 atelocollagen (5 mg/ml, pH 3.0, Koken, Tokyo, Japan), and 0.1, 1, or 10 mM glutathione (Sigma) were added to the medium. Atelocollagen solution diluted with HCl (pH 3.0) was added to the medium at 1 % (v/v) of the medium. Two to three sheets were prepared for each condition.

Measurement of sheet size and wet weight

A sheet, which was taken out from the well using a spatula, was wiped using tissue paper to remove excess water and weighed. Pictures of the sheet and ruler, which were put side by side, were taken using a digital camera (Canon). The pictures of cell sheets were taken from the top and side. The diameter and thickness of the sheets were measured from the pictures using Image J software.

Determination of cell number in the cell sheet

Disc-shaped cell sheets were dissociated at 37 °C for 40 min using 5 g/l collagenase (Wako), 5 g/l collagenase type II (Worthington Biochemical, Lakewood, NJ, USA), and 5 g/l trypsin (Sigma). Cell number was determined by the trypan-blue method after dissociation.

Determination of ECM accumulation

Sulfated glycosaminoglycan (sGAG) was extracted from cell sheets by immersing the sheets in an extraction solution [4 M guanidine hydrochloride (Wako), 10 mM Na₂EDTA (Dojindo, Kumamoto, Japan), 0.1 M ε-aminocaproic acid (Kishida Chemical, Osaka, Japan), 5 mM benzamidinium hydrochloride

(Wako)] for 48 h. The level of extracted sGAG was determined by the 1–9-dimethylmethylene blue (DMMB; Sigma) binding method using chondroitin sulfate C as the standard. The results are shown in two ways; total sGAG amount per sheet and the ratio of sGAG weight (μg) to the total wet weight of a construct (mg) as the index of cell activity and mechanical characteristic of the sheets, respectively.

The sheets were immersed in guanidine hydrochloride solution (3 M in 0.05 M Tris–HCl buffer) with shaking overnight at 4 °C and were then centrifuged (10,000 rpm, 3 min, 4 °C). The resulting precipitate was washed twice with cold water, suspended in acetic acid (0.05 M in 0.5 M NaCl; pHs 2.9–3.0) with pepsin solution (Wako, 10 mg/ml in 0.05 M acetic acid) with shaking for 48 h at 4 °C. The resulting precipitate was suspended in a buffer (0.1 ml, 1.0 M Tris, 2.0 M NaCl, 50 mM CaCl_2 ; pHs 7.8–8.0) and pH was adjusted to 8.0 with NaOH (1 N). The mixture was incubated with pancreatic elastase solution (Wako, 1 mg/ml in the buffer) overnight at 4 °C. After centrifugation (10,000 rpm, 5 min), the type 2 collagen concentration in the extracted supernatant was determined by ELISA (Chondrex, Redmond, WA, USA) following the manufacturer's instruction. The results are shown in two ways; total type 2 collagen amount per sheet and the ratio of type 2 collagen weight (μg) to the total wet weight of a construct (mg) as the index of cell activity and mechanical characteristic of the sheets, respectively.

Histological staining

The constructs were washed with PBS, fixed in 10 % formalin solution, and embedded in paraffin. The constructs were cut into 4- μm -thick sections, deparaffinized with xylene, and rehydrated with ethanol. The sections were stained with propidium iodide (PI; Sigma), alcian blue (Wako), and toluidine blue solution (Wako) for histological analysis.

Gene expression analysis

Disc-shaped cell sheets were dissociated at 37 °C for 40 min using 5 g/l collagenase, 5 g/l collagenase type II, and 5 g/l trypsin. Total RNA was extracted from the cells using a GenElute™ Mammalian Total RNA Miniprep kit (Sigma). The extracted total RNA was treated with DNase-I (Takara) to degrade genomic DNA. DNase-treated RNA was used to produce cDNA

using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA, USA) and a thermal cycler (Applied Biosystems 2720). Quantitative reverse transcription PCR (qRT-PCR) was performed with cDNA using the AmpliTaq Gold® 360 Master Mix (Applied Biosystems), appropriate primers, a TaqMan probe, and the ABI PRISM 7700 system (Applied Biosystems). PCR cycles consist of 10 min at 95 °C (1 cycle), 15 s at 95 °C, 1 min at 60 °C (55 cycles), and 7 min at 72 °C (1 cycle). For all samples, aggrecan, type 2 collagen, type 1 collagen, type 10 collagen, and MMP-13 mRNA expression levels were normalized by β -actin mRNA expression level. The primers and probes for aggrecan and type 2 collagen are shown in Table 1. Those for β -actin (Hs01060665_g1), type 1 collagen (Hs00164004_m1), type 10 collagen (Hs00166657_m1), and MMP-13 (Hs00233992_m1) were purchased from Applied Biosystems. The cDNA prepared using RNA isolated from primary human chondrocytes in the articular cartilage, which was obtained from the donors undergoing joint surgery with their informed consent was employed as a positive control for PCR analysis. The expression level of each gene was determined from the standard curve using control cDNA, as follows:

$$\text{Gene expression level (\%)} = \frac{\frac{\text{quantity of target gene (cell sheet)}}{\text{quantity of } \beta\text{-actin (cell sheet)}}}{\frac{\text{quantity of target gene (control)}}{\text{quantity of } \beta\text{-actin (control)}}} \times 100$$

The ratio of the expression level of each gene with additives to that without additives is shown.

Table 1 Sequences used in PCR

Gene	Sequence
Aggrecan	
F primer	5'-AGTCCTCAAGCCTCCTGTACTCA-3'
R primer	5'-GCAGTTGATTCTGATTACAGTTTC-3'
Probe	5'-ATGCTTCCATCCCAGCTTCTCCGG-3'
Col2A1	
F primer	5'-CGCTGTCCTTCGGTGTCTAG-3'
R primer	5'-CCTTGATGTCTCCAGGTTCTCC-3'
Probe	5'-CCAGGATGTCCGCAACCAGGA-3'
Col1A1	Hs00164004_m1
Col10A1	Hs00166657_m1
MMP-13	Hs00233992_m1
β -actin	Hs01060665_g1

Statistical analyses

Two to three cell sheets were prepared for each condition. ECM determination and RT-PCR were

carried out twice and once, respectively. Data are presented as average $\pm$ SD. Two-tailed Student's *T* test was carried out for statistical analysis of data. Values of $p < 0.05$ were considered to be statistically significant.

Fig. 1 Effect of VCP on MSC sheet culture. Top (a–d) and side (e–h) views of cell sheets. One scale indicates 1 mm

Fig. 2 Effect of VCP on MSC sheet culture. Wet weight (a), cell number (b), sGAG content (c), type 2 collagen content in cell sheets (e). sGAG (d) and type 2 collagen contents were

normalized by sheet wet weight (f). Individual data ($n = 2$) are shown instead of average. *N.D.* not detected

Fig. 3 Histological staining of sections. The sections of an MSC sheet cultivated with VCP added at common concentration (25 µg/ml) (**a**, **c**, **e**) and newly proposed concentration (250 µg/

ml) (**b**, **d**, **f**) were stained with PI (**a**, **b**), alcian blue (**c**, **d**), and toluidine blue (**e**, **f**). The scale bar indicates 200 µm

Results

Effect of ascorbic acid on MSC sheets

To determine the effect of ascorbic acid, MSCs (donor A) were cultivated for 3 weeks in the cell culture insert using the chondrogenic medium containing 25, 50, 250, and 2500 µg/ml VCP. There was no significant difference in sheet diameter (approximately 6 mm) (Fig. 1a–d) and thickness (approximately 0.3 mm)

between culture conditions (Fig. 1e–h). No significant difference was observed in the appearance of the cell sheets. The wet weight of cell sheets at 3 weeks increased as the concentration of VCP increased to 250 µg/ml (Fig. 2a, no significant difference). The cell number and sGAG content in the cell sheets were not affected by VCP except at a VCP concentration of 2500 µg/ml, while the differences were not statistically significant (Fig. 2b–d). On the other hand, the amount and density of type 2 collagen in the cell sheets

markedly increased following the addition of VCP (Fig. 2e, f, non-significant). There were no significant differences in cell viability in cell sheets between the different culture conditions (90–91 %) (Data not shown).

In order to compare the new condition (250 μg VCP/ml) to the common condition (25 μg VCP/ml), the cell sheets to which VCP was added at 25 and 250 $\mu\text{g}/\text{ml}$ were histologically stained (Fig. 3). PI staining and histological staining with alcian blue and toluidine blue showed comparable cell numbers and sGAG contents in both cell sheets with VCP added at 25 and 250 $\mu\text{g}/\text{ml}$, which were in agreement with the results shown in Fig. 2.

Effect of glutathione on MSC sheets

To determine the effect of glutathione, MSCs (donor A) were cultivated for 3 weeks in the cell culture insert using the chondrogenic medium containing 0, 0.1, 1, 10,

10 mM glutathione. There was no significant difference in sheet diameter (approximately 6 mm) between culture conditions. However, the thickness of cell sheets was slightly decreased by the addition of glutathione [approximately 0.3 mm (0 mM) and 0.2 mm (10 mM)]. The wet weight and type 2 collagen accumulation level in the cell sheets apparently decreased following the addition of glutathione, whereas the content of sGAG in the cell sheets slightly increased (Fig. 4). Significant differences were observed in wet weight and GAG content [Wet weight: $p = 0.0006$ (0.1 vs 0 mM), $p = 0.0003$ (1 vs 0 mM), $p = 0.00003$ (10 vs 0 mM), GAG content: $p = 0.003$ (0.1 vs 0 mM), $p = 0.02$ (10 vs 0 mM)].

Effect of type 1 atelocollagen on MSC sheets

To determine the effect of type 1 atelocollagen, MSCs (donor B) were cultivated for 3 weeks in the cell culture insert using the chondrogenic medium

Fig. 4 Effect of glutathione on MSC sheet culture. Wet weight (a), sGAG content (b), and type 2 collagen content (d) in cell sheets. sGAG (c) and type 2 collagen (e) contents were normalized by sheet wet weight ($n = 3$, average $\pm$ SD). * $p < 0.05$

Fig. 5 Effect of type 1 atelocollagen on MSC sheet culture. Wet weight (**a**), sGAG content (**b**), and type 2 collagen content (**d**) in cell sheets. sGAG (**c**) and type 2 collagen (**e**) contents were normalized by sheet wet weight. All individual data ($n = 2$) were shown instead of average

Fig. 6 Side views of cell sheets in Fig. 7. One scale indicates 1 mm

containing 0, 0.05, 0.5, 5, and 50 µg/ml type 1 atelocollagen. There was no significant difference in sheet diameter (approximately 6 mm) and thickness (approximately 0.6 mm) between culture conditions. No significant difference was observed in the appearance of cell sheets. The amount of sGAG in the cell sheets slightly increased following the addition of collagen, whereas the wet weight of cell sheets was not affected (Fig. 5a–c). In contrast, the amount of type 2 collagen in the sheets increased following the addition of type 1 atelocollagen at all concentrations except 50 µg/ml collagen (Fig. 5d, e, the difference was statistically not significant).

Effect of co-addition of ascorbic acid and type 1 atelocollagen

To confirm the synergistic effect of VCP and collagen on type 2 collagen accumulation in the sheets, both VCP (250 µg/ml) and type 1 atelocollagen (5 µg/ml) were added to the sheet culture (MSC; donor A). There was no significant difference in sheet diameter (approximately 6 mm) between culture conditions. The thickness of cell sheets was slightly changed by the addition of VCP and type 1 atelocollagen [approximately 0.35 mm (control), 0.3 mm (COL), 0.35 mm (VCP), and 0.4 mm (C + V) (Fig. 6a–d)].

Fig. 7 Synergistic effect of VCP and type 1 atelocollagen on MSC sheet culture. Type 1 atelocollagen (COL, 5 μg/ml), VCP (VCP, 250 μg/ml), and both (C + V) were added to the MSC sheet culture. Wet weight (a), sGAG content (b), and type 2

collagen content (d) in cell sheets. sGAG (c) and type 2 collagen (e) contents were normalized by sheet wet weight (n = 3, average ± SD). **p* < 0.05

The wet weight of the cell sheets increased in the culture with VCP and in that with both VCP and collagen (Fig. 7a, [wet weight: *p* = 0.003 (COL vs no addition (N)); *p* = 0.027 (VCP vs N); *p* = 0.0006 (C + V vs N)]. The GAG density of the cell sheets increased only in the culture with collagen added (Fig. 7c, [GAG density: *p* = 0.002 (COL vs N)]). The amount and density of type 2 collagen increased following the addition of both VCP and collagen (twofold and fourfold increases, respectively). Moreover, the addition of both VCP and collagen markedly increased the amount and density of type 2 collagen by sixfold and eightfold, respectively (Fig. 7d, e, [Type 2 collagen content: *p* = 0.008 (VCP vs N); *p* = 0.026 (C + V vs N)], [Type 2 collagen density: *p* = 0.014 (VCP vs N); *p* = 0.028 (C + V vs N)]).

The same culture experiment as that shown in Fig. 7 was separately performed and the gene expression levels were determined at 3 weeks (MSCs; donor

A) (Fig. 8). Type 1 and type 10 collagen gene expression levels did not increase following the addition of collagen and/or VCP (Fig. 8c), whereas there was a slight increase in type 10 collagen expression level following the addition of both collagen and VCP (C + V in Fig. 8d, the difference was statistically non-significant). The expression levels of aggrecan increased following the addition of both collagen and VCP (C + V in Fig. 8a, the difference was statistically non-significant). Although the expression level of type 2 collagen increased by twofold following the additions of only VCP and that of both collagen and VCP, the addition of only collagen did not result in the increase in the expression level of type 2 collagen (Fig. 8b, the difference was statistically non-significant). In contrast, the addition of collagen and VCP decreased the expression level of MMP-13 to approximately 70 % of the control, respectively. Moreover, the addition of both VCP

Fig. 8 Effect of VCP and type 1 atelocollagen on chondrogenic gene expressions in MSC sheet culture. Type 1 atelocollagen (COL, 5 µg/ml), VCP (VCP, 250 µg/ml), and both (C + V) were added to the MSC sheet culture. Gene expression levels of

aggrecan (a), type 2 collagen (b), type 1 collagen (c), type 10 collagen (d), and MMP-13 (e). The ratio of each data to the average of control data without additives is shown. Individual data (n = 2) are shown instead of average

and collagen markedly decreased the expression level of MMP-13 to 35 % of the control (Fig. 8e, statistically non-significant).

Discussion

The increase in the accumulation of type 2 collagen by the addition of VCP was demonstrated in both figures [Fig. 2 (n = 2) and Fig. 7 (n = 3)], while the increase could not be confirmed statistically by the experiment shown in Fig. 2 due to the small sample number of 2. Although ascorbic acid is usually added at a concentration of 50 µg/ml, we found a significant increase in type 2 collagen accumulation level following the addition of VCP at a higher concentration (250 µg/ml) (Fig. 2). Ascorbic acid has been reported to have antiapoptotic action (Siow et al. 1999; Yun et al. 2001). However, the cell number (Fig. 2)

and nucleus density observed after PI staining (Fig. 3) in the cell sheets were not increased by VCP. Although glutathione, as well as ascorbic acid, has an antioxidant action in cells (Hong et al. 2001; Meister 1992; Yun et al. 2001), the addition of glutathione to the chondrogenic medium decreased the wet weight of cell sheets and type 2 collagen content in a dose-dependent manner (Fig. 4). Therefore, we considered that the reason for the increase in type 2 collagen content in the cell sheets following VCP addition might not only be due to the antiapoptotic action of VCP.

It was reported that ascorbic acid induces and enhances chondrogenic differentiation of ATDC5 cells through the activation of the ERK pathway (Temu et al. 2010). Collagen is also known to bind to integrin and enhances ERK signaling (Chetoui et al. 2006). Therefore, we added type 1 atelocollagen or VCP to chondrogenic medium, which resulted in the

increases in sGAG and type 2 collagen accumulation level (Fig. 5), while the addition did not affect the expression levels of aggrecan and type 2 collagen genes (Fig. 8a, b). In contrast, the addition of collagen markedly decreased the expression levels of MMP-13 to approximately 70 % of the control (Fig. 8e). Moreover, the addition of VCP increased type 2 collagen gene expression level and decreased MMP-13 gene expression level (Fig. 8b, e). Consequently, it was suggested that the increase in type 2 collagen accumulation levels following the addition of collagen or/and VCP might be due to the inhibition of the degradation of type 2 collagen and the stimulation of type 2 collagen gene expression.

There was no significant difference in type 2 collagen gene expression level between the addition of only VCP and that of both VCP and collagen (Fig. 8b). However, the expression level of the MMP-13 gene in the sheets with both of VCP and collagen added was markedly lower than those in the sheets with the addition of only collagen or only VCP (Fig. 8e). Consequently, there might be a synergistic effect of VCP and collagen resulting in the inhibition of MMP-13 gene expression. These findings indicate the synergistic effect of ascorbic acid and collagen on type 2 collagen accumulation in MSC sheet culture.

In conclusion, the addition of ascorbic acid and/or collagen at a markedly higher concentration than in previous reports increased the type 2 collagen accumulation level in the MSC sheets by up to eightfold. This effect might not only be through the enhancement of collagen synthesis by promoting mRNA synthesis but also through the suppression of collagen degradation by the suppression of MMP-13 gene expression. The enhancement of ECM synthesis and the suppression of MMP-13 gene expression might be useful for cartilage regeneration. The clinical applications of the additives VCP and type 1 atelocollagen to cartilage regeneration might be assessed in the future.

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