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# Tissue Development and Mechanical Property in the Regenerated-Cartilage Tissue

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Additional information is available at the end of the chapter

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## 1. Introduction

Vertebrates have multiple synovial joints, and these joints in humans enable a number of activities of daily life. In particular, the hip, knee, shoulder, elbow, and ankle are all synovial joints with synovium in which the secretes synovial fluid. The articular surface is covered with smooth hyaline cartilage, which forms the part of the skeletal system that is notably different from mineralized bone in both function and histological composition [1]. The formation of animal cartilage from mesenchyme occurs in numerous areas of the embryo, such as the skull, limbs, and spine. This tissue contains chondrocytes which synthesize and maintain extracellular matrix (ECM), which is composed of a dense network of collagen molecules and proteoglycans [2]. There are three types of cartilage; fibrocartilage, elastic cartilage, and hyaline cartilage. Fibrocartilage contains regions of organized fibrous tissue, containing type I collagen in addition to the normal type II collagen. It is found in the annulus fibrosus of the intervertebral discs, meniscus and temporomandibular joints. Elastic cartilage contains additional elastin fibers and is a type of cartilage present in the ear, larynx, and epiglottis. Finally, hyaline cartilage coats the articular surfaces of bone epiphyses and is composed of individual chondrocytes bound together by the ECM. The major constituent of the hyaline cartilage is held in place by proteoglycan (about 10 % of wet weight) and type II collagen (10–20 % of wet weight), which forms a meshwork with high tensile strength [3–5].

Steric and electrostatic interactions of these ECM molecules in the hyaline cartilage occur between the cationic collagen fibers and the anionic proteoglycans to provide a highly charge in a neutral pH environment. In particular, since the proteoglycan is highly negatively charged, it is not only cross-liked between collagen, but also combined with many water molecules, leading to high extracellular osmolality within the cartilage. Consequently, cartilage is as formed as a soft-tissue-bearing element with high viscoelasticity [6]. The mechanical function of the hyaline cartilage is joint lubrication and shock absorption, and articular cartilage has a very low friction coefficient (0.02) [7] with smoother movement than most modern artificial joint replacements.

Although adult articular cartilage is a remarkable load bearing system, it lacks the ability to repair itself under conditions of wear and tear or traumatic injury, leading to osteoarthritis (OA). This lack of self-maintenance is due to its avascular, aneural, alymphatic and almost nonimmunogenic properties, as well as its nourishment entirely via diffusion from synovial fluid. Diseases of the cartilage are a major health problems, especially in industrialized countries with long life expectancy. At present, there is no established therapy of cell-assisted tissue regeneration for sufficiently reliable and durable replacement of damaged articular cartilage [8–11]. In recent years, however, tissue engineering has shown promise toward the treatment of OA, enabling researchers to produce functional replacements for diseased cartilage [12, 13]. Developments in therapeutic strategies for damaged cartilage treatment have increasingly focused on the promising technology of cell-assisted repair and proposing the use of autologous chondrocytes or other cell types to regenerate articular cartilage [10, 11].

Little knowledge is available for establishing a suitable design strategy for reconstructing cartilage by tissue engineering to match the mechanical properties of natural tissue. It is therefore necessary to understand the relationship between degree of development of the ECM network and the macroscopic mechanical properties of the cultured construct for producing regenerated cartilage. Because the ECM meshwork is formed chiefly by type II collagen, growth of the collagen network likely plays the most important role in the mechanical characteristics of the overall three-dimensional construct (e.g., Young's modulus and compressive strength). If ECM network does not interconnect among chondrocytes in the cell-scaffold material, it is no relevant to entire mechanical property of the construct. In such a case, this mechanical property will be the same as that of the scaffold material without chondrocytes. This means that less mechanical property increases if the ECM synthesizes a lot. We therefore investigated the relationship between development of the ECM network and the macroscopic mechanical property of the cultured construct using vitamin C (VC) to control collagen synthesis [14].

VC is an important water-soluble antioxidant and enzyme cofactor of collagen synthesis in plants and animals [15] and the importance of VC in collagen synthesis is well-known as described below [16–18]. Procollagen molecules, which are collagen precursors, undergo multiple steps of post-translational modification. After translocation of the growing polypeptide chains of procollagens into the rough endoplasmic reticulum, hydroxylation of proline residues is catalyzed by prolyl 4-hydroxylase (P4H). This catalytic reaction requires ferrous ions, 2-oxoglutarate, molecular oxygen, and ascorbic acid (AsA), a kind of VC, as cofactors. The hydroxylation of proline residues increases the stability of the triple helix and is a key element in its folding. P4H requires an unfolded chain as a substrate. The C-propeptides have an essential function in the assembly of the three  $\alpha$ -chains into trimeric collagen monomers. The formation of triple helices starts from the alignment of the C-terminal domains of the three  $\alpha$ -chains and proceeds to the N-terminal. Premature association between procollagen is thought to be prevented by heat shock protein 47 [18] and by collagen-modifying enzymes until biosynthesis of the individual chain is complete. In the collagen-secretion process, proline hydroxylation is caused by oxidation of P4H-bound ferrous iron, which must be reduced to P4H-bound ferric iron by AsA to reinvigorate P4H activity for maintaining the proline hydroxylation process. Thus, the collagen biosynthesis needs AsA to reduce oxidized P4H-bound iron.

Many studies have reported the relationship between collagen content in the tissue and mechanical properties [19–21] and the effects of VC on several cell functions (e.g., cytotoxicity

and redox) [15, 22–25]; however, little knowledge is available on the development of the ECM network in relation to the mechanical properties of the cultured construct. In this chapter, we demonstrate the influence of collagen network growth on the macroscopic mechanical properties of regenerated cartilage using a chondrocyte-agarose construct, and we briefly explain how expansion of the network within a tissue affects to the mechanical properties. Here we used two types of VC: AsA, the acidic form; and ascorbic acid 2-phosphate (A2P), the non-acidic form. In addition, after applying uniaxial compressive strain to the tissue model using a purpose-built bioreactor, we described the different ways of developing the ECM in the construct by dosing the culture medium with AsA or A2P.

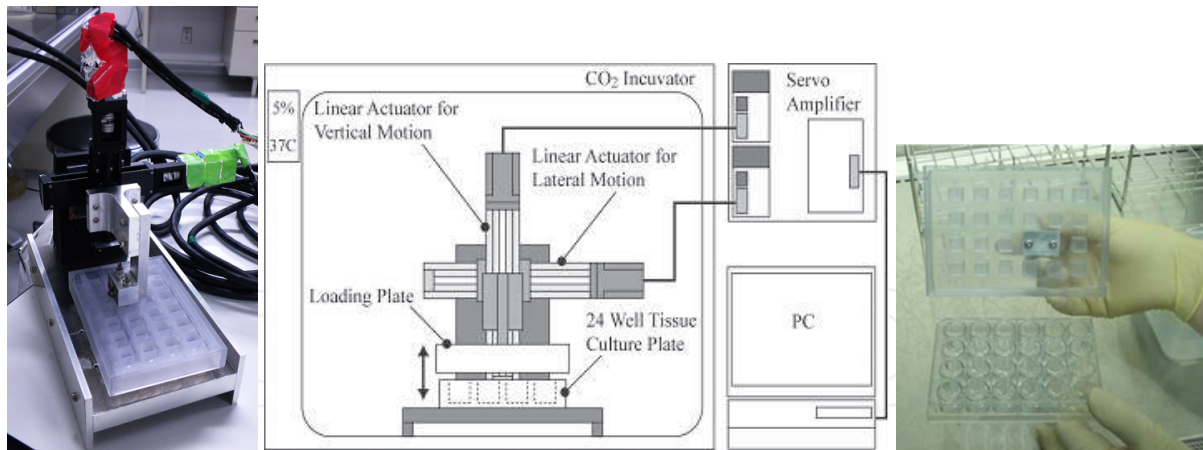
## 2. Materials & methods

### 2.1. Sample preparation and culturing

Primary bovine chondrocytes were isolated from metacarpophalangeal joints of steers purchased from a meat center in Fukuoka city, Japan using a sequential enzyme digestion method [26]. Full-thickness articular cartilage tissue was harvested from the proximal articular surface of the metatarsal bone and finely diced with a scalpel. The finely diced cartilage was enzymatically digested with 25 unit/mL protease solution (P8811, Sigma, St Louis, MO) for 3 hours, and subsequently with 200 unit/mL collagenase solution (C7657, Sigma) for 18 hours at 37 degree C. Both enzyme solutions were prepared in sterile tissue culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM; D5921, Sigma) supplemented with 20 v/v% Fetal Bovine Serum (FBS; 10437-028, Gibco, CA), 2 mM L-glutamine (G7513, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 unit/mL penicillin, 100  $\mu$ g/mL streptomycin, 0.25  $\mu$ g/mL amphotericin B (161-23181, Wako), 20 mM hepes (H0887, Sigma), and 0.85 mM L-ascorbic acid (AsA; A5960, Sigma). The supernatant of the resultant solution was centrifuged to separate chondrocytes at  $40\times g$  for 5 min. The resultant cell pellet was washed twice with fresh culture medium, and cell number and cell viability were determined by trypan blue assay. In this study, Sigma Type VII agarose (A6560, Sigma) was used to prepare a chondrocyte-agarose construct. The agarose powder was dissolved in Earle's balanced salts solution (EBSS; Sigma) at twice the required final concentration (1 w/v%) and mixed with an equal volume of the cell suspension to yield the desired agarose concentration with a final cell density of  $1 \times 10^7$  cells/mL. The molten cell-agarose solution was poured into an acrylic mold and quenched to gel at 4 degree C for 30 min to create cylindrical constructs with a diameter of 4 mm and a height of 2.5 mm. The resultant cell-agarose constructs were placed into a 24-well culture plate and subsequently cultured with 1 mL culture medium in a humidified tissue culture incubator controlled at 37 degree C and 5 % CO<sub>2</sub>. The culture medium was exchanged every two days. Several culture media with different VC concentrations, from 0.64 to 6.4 pmol/10<sup>9</sup> cells were prepared and used to evaluate the effect of AsA and A2P Salt (Wako) concentrations. The culture medium without VC was also used as a control. Media are denoted abbreviated as AsA(0.64), AsA(2.2), AsA(3.2), A2P(3.2), and A2P(6.4) hereafter in this paper.

### 2.2. Mechanical stimulation

After 1 day of free-swelling culture, the constructs in the experiment group were subjected to uniaxial compression within a purpose-built bioreactor system as shown in Fig. 1. The system enables the application of strain independently in each vertical and horizontal direction to



**Figure 1.** Photograph (left) and Schematic drawing (center) of the mechanical loading system mounted within tissue culture incubator and appearance of the loading platen with 22 plungers coupled with a 24 well culture plate (right).

individual constructs using a 24-well plate in a commercially available incubator. Movements was controlled with respective linear variable displacement transducers and linear guide actuators. Strain was applied to the individual constructs through a loading plate, which was attached to the actuator via a jig. Uniaxial cyclic compression up to a maximum amplitude of 15 % was applied with a triangular waveform at a frequency of 1 Hz for 6 hours and subsequently off-loaded with the platen resting on each construct for the subsequent 18 hours. Control constructs were cultured in contrast with both upper- and lower-platens to diffusion through its sides alone.

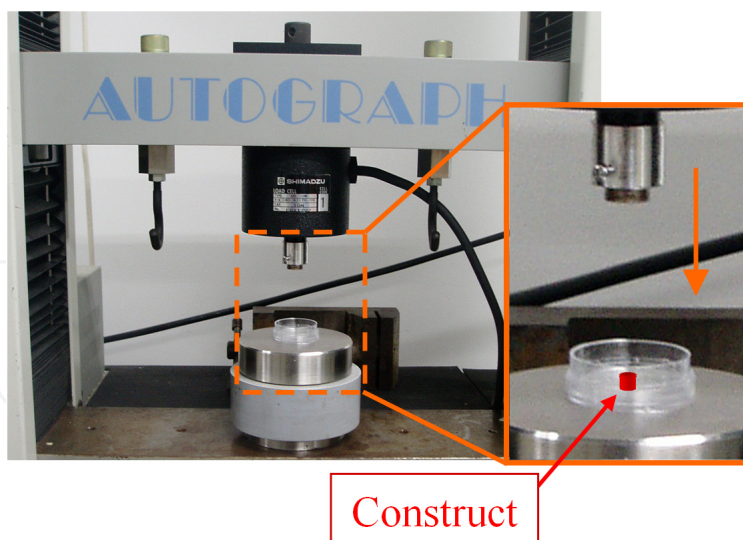
### 2.3. Mechanical testing

To examine the influence of chemical and physical stimulation on the mechanical properties of the constructs, cell-agarose constructs were subjected to unconfined compression while immersed in culture medium at room temperature. Individual constructs were tested after culture periods of 1, 8, 15, and 22 days. Mechanical tests were performed with an impermeable stainless steel plunger at a strain rate of 0.5 mm/min up to a strain of 10%, while the load was recorded with a 10-N load sensor, as shown in Fig. 2. The tangent modulus of the construct was calculated from the slope of a straight-line approximation of the stress-strain curve with a range of 0–15 % strain using the least-square method.

### 2.4. Immunohistology

Separate constructs were used for trichrome immunofluorescence observation to examine the morphological characteristics of the elaborated ECMs, in particular type I collagen, type II collagen, and chondroitin sulphate. After the prescribed culture periods, representative constructs were cut into slices with a thickness of approximately 1 mm using a knife. The slices were washed in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free phosphate-buffered saline (PBS(-)) and subsequently incubated in PBS(-) + 1 w/v% bovine serum albumin (BSA; Wako) for 30 min at 37 degree C. These slices were incubated in PBS(-) containing the three monoclonal antibodies (bovine IgG1 isotype anti-type I collagen, Funakoshi, Tokyo, Japan; embryonic chicken IgG2a isotype anti-type II collagen, Funakoshi; mouse IgM isotype anti-chondroitin sulphate, Sigma)





**Figure 2.** Photograph of compression test for determining the tangent modulus

for 90 min at 37 degree C to primarily label the collagens and the proteoglycan at once. The slices were then washed three times in PBS(-) for 10 min and incubated in PBS(-) containing the three secondary antibodies corresponding to each of the primary antibodies (Alexafluor 350-conjugated anti-mouse IgG1 antibody, A21120; Alexafluor 488-conjugated anti-mouse IgG2a antibody, A11001; Alexafluor 568-conjugated anti-mouse IgM antibody, A21043, Invitrogen) for 60 min at 37 degree C to fluorescently visualize the labeled ECM molecules within the cultured cell-agarose construct. The fluorescently stained specimen was mounted on the coverslip and observed using a confocal laser scanning microscope (CLSM; Eclipse; Nikon Corp., Tokyo, Japan).

## 2.5. Statistical analysis

Result of the tangent modulus were expressed as the mean  $\pm$  sample standard deviation ( $SD$ ). The significance of the difference between each experimental group was assayed using the two-tailed Welch's  $t$ -test. The degree of freedom is abbreviated as  $df$ . If the results of pairwise comparisons between two groups were  $P < 0.01$ ,  $d > 1$  and  $(1 - \beta) > 0.8$  simultaneously, we judged the difference in the tangent modulus as significant, where  $P$ : level of significance;  $d$ : Cohen's  $d$ , a kind of the effect size [27];  $(1 - \beta)$ : the power of test as calculated using the R language.

## 3. Results

### 3.1. Effect of VC on mechanical properties of the tissue under free-swelling culture conditions when added to the medium

#### 3.1.1. Influence on mechanical properties

The tangent modulus ( $E$ ) of the four experiment groups (without VC, AsA(0.64), AsA(2.2), and AsA(3.2)) was determined at 22 days of culture in Fig. 3. The tangent moduli of the high AsA dose groups, AsA(2.2) and AsA(3.2), were significantly higher than those of the without AsA group. Moreover, we measured the temporal growth of the construct's tangent modulus to

determine the relationship between development of the ECM and mechanical properties. The tangent moduli of all experiment groups after 1 day of culture were almost identical (results not shown) and were equivalent to the tangent modulus of cell-free agarose gel ( $12.5 \pm 0.9$  kPa,  $n = 10$ ). The measured tangent modulus ( $E$ ) was normalized with the tangent modulus at day 1 ( $E_0$ ) and plotted against the culture periods in Fig. 4. It was evident that the tangent modulus increased with increasing culture period. These results clearly indicate that the growth rate of the tangent modulus is dependent on the AsA concentration of the culture medium. Thus, the tangent modulus of the high AsA dose groups, AsA(2.2), and AsA(3.2), increased faster than that of the low AsA dose group, AsA(0.64).

### 3.1.2. Histological observation

Typical immunofluorescence images of elaborated ECMs (type I collagen, type II collagen and chondroitin sulfate) after 22 days of culture were compared as shown in Fig. 5. It is clear that in the low-VC concentration groups (without VC and AsA(0.64)), only a small amount of ECM which is consisting of chondroitin sulfate was present, as indicated in Fig. 5(A) and 5(B). By contrast, abundant ECM, chiefly type II collagen, was observed in the constructs of AsA(2.2) and AsA(3.2), as indicated by the arrow-heads in Fig. 5(C) and 5(D). Then, the broadened collagen network interconnected the chondrocytes to create an extended network which exceeded the field of view in the photograph.

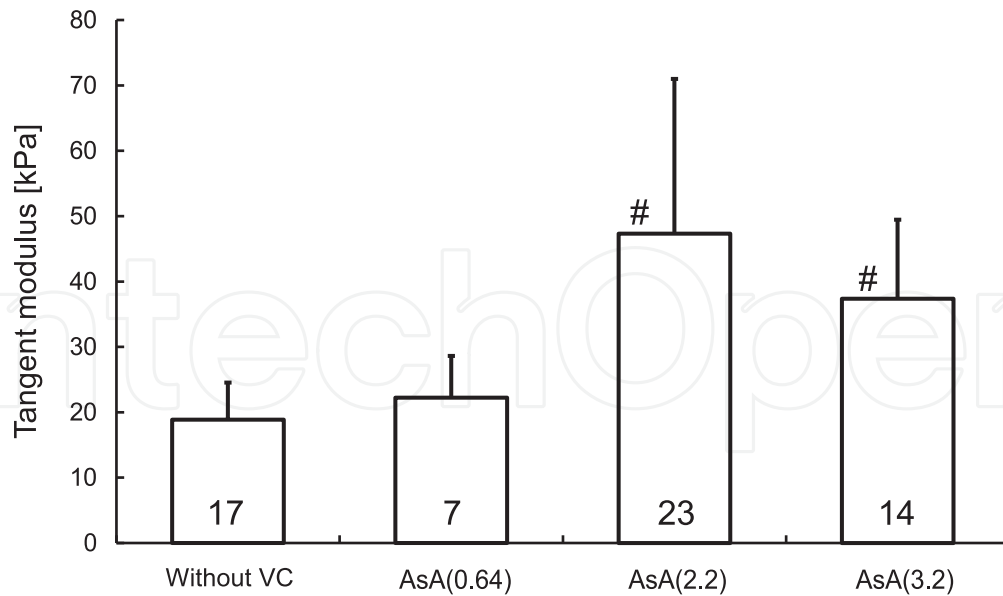
## 3.2. Influence of mechanical stimulation on the development of the ECM and mechanical properties

### 3.2.1. Measurement of mechanical properties

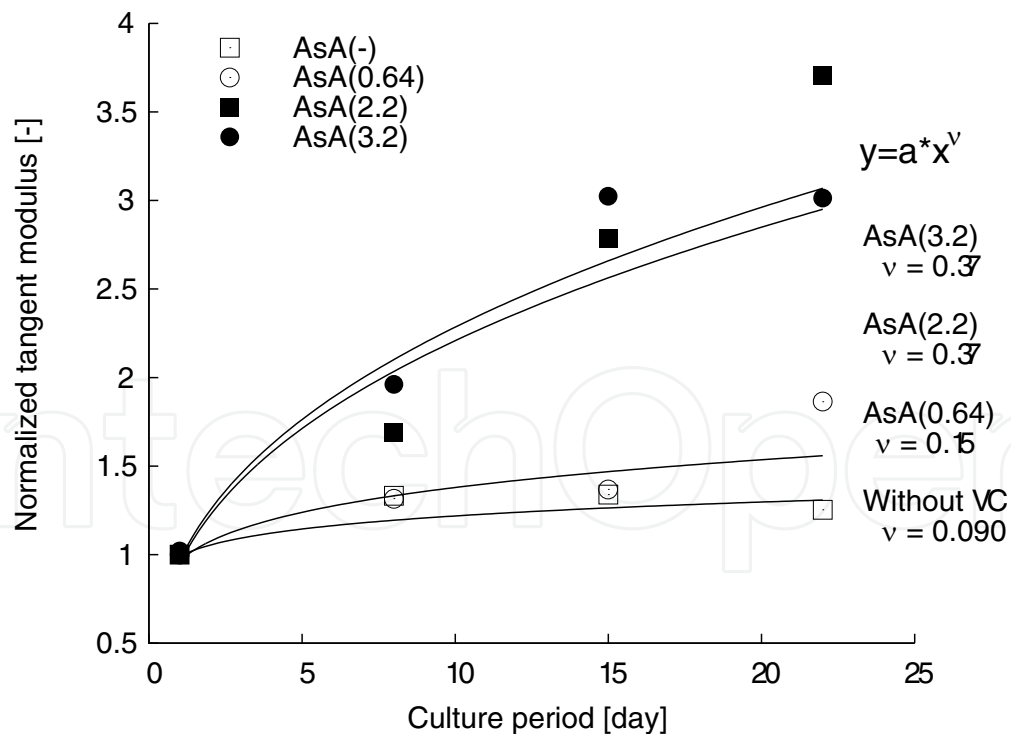
To evaluate the influence of mechanical stimulation on the cultured constructs, the tangent moduli of each group after 22 days of culture was measured in Fig. 6. A significant difference was noted between the without VC and AsA(2.2) groups compared with the three free-swelling groups, the latter revealing an increased tangent modulus. When applying cyclic compression to the construct with dosing the culture medium with VC, no clear difference was observed between free-swelling and compression AsA groups. By contrast, both tangent moduli of compression A2P dose group (A2P(6.4)) were higher than those of the free-swelling A2P(6.4) group. Each tangent modulus of control groups with A2P was ranked between free-swelling and compression groups. No differences were evident between the three compression groups ( $df = 16, P = 0.47, d = 0.32, (1 - \beta) = 0.030$ ).

### 3.2.2. Histological observation

Figs. 7 and 8 show immunofluorescent images at low- and high-magnification and reveal the ECM distribution (type I and II collagen and chondroitin sulfate) after 22 days of culture, with the high-magnification images taken near the center of the construct. It is clear that in the absence of VC in the culture medium, there was only a limited quantity of ECM, as indicated in Fig. 7(A) and 8(A). By contrast, in the free-swelling group (AsA(2.2)), there was fairly uniform distribution of ECM across the construct (Fig. 7(B)) which, at high magnification, revealed a collagen network that interconnected between chondrocytes, as indicated by the arrows in Fig. 8(B). The low-magnification images for each of the A2P dose groups revealed similar distribution in ECM across the constructs (Figs. 7(C)–(E)). In the high-magnification



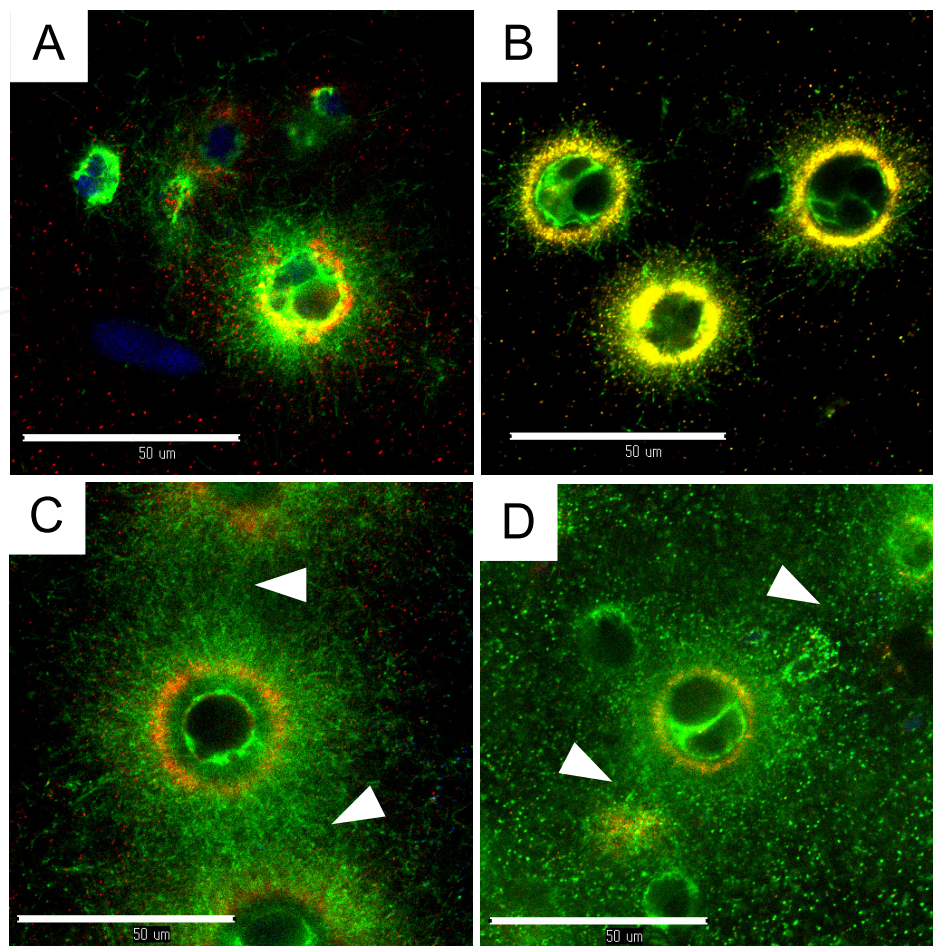
**Figure 3.** Comparison of tangent modulus after 22 days culture period. Each number in a column is cultured sample number, and each sample represents an individual culture. Error bar means *SD*. Sharps (#) was shown statistical significant respective as compared to control group and between each groups. #, \*:  $d > 1$ ,  $P < 0.01$ ,  $(1 - \beta) > 0.8$ .



**Figure 4.** Comparison of normalized tangent modulus with the tangent modulus at day 1 ( $E_0$ ).

images, type II collagen molecules were appeared to interconnect between chondrocytes, as indicated by arrows in Fig. 8(E). These results demonstrate that the compression group of





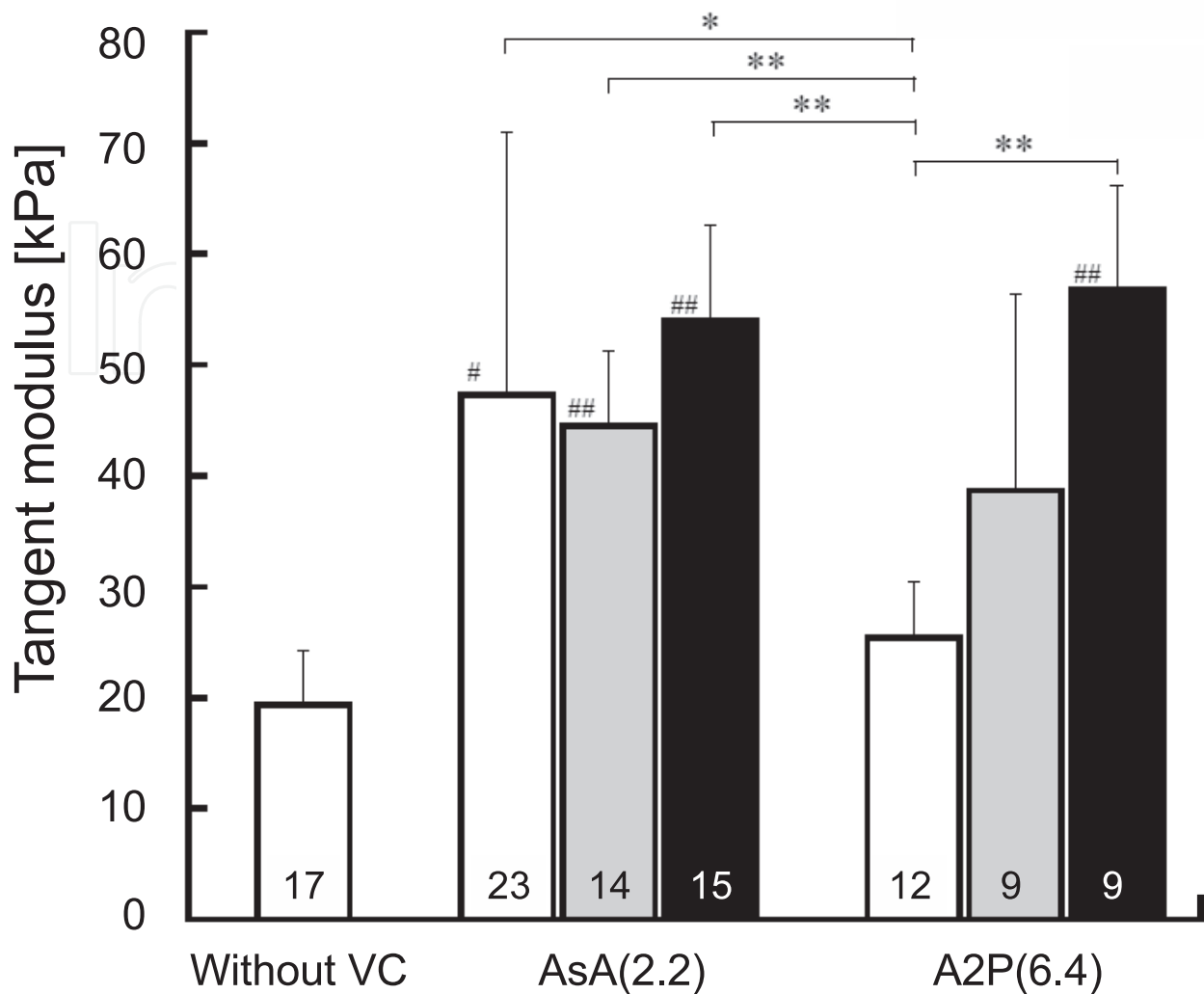
**Figure 5.** Fluorescence images of ECMs: blue: type I collagen; green: type II collagen; red: chondroitin sulphate. Culture period was 22 days. (A): without VC; (B): AsA(0.64); (C): AsA(2.2); (D): AsA(3.2). Each trichrome stained sample represents an individual culture. Scale bar represents 50  $\mu\text{m}$ .

A2P(6.4) is associated with more of the collagen network than the corresponding free-swelling group. In addition, the distribution of chondroitin sulfate was clearly observed to be restricted to the peripheral regions of the chondrocytes.

## 4. Discussion

### 4.1. Effect of VC on mechanical properties of the tissue under free-swelling culture conditions when added to the medium

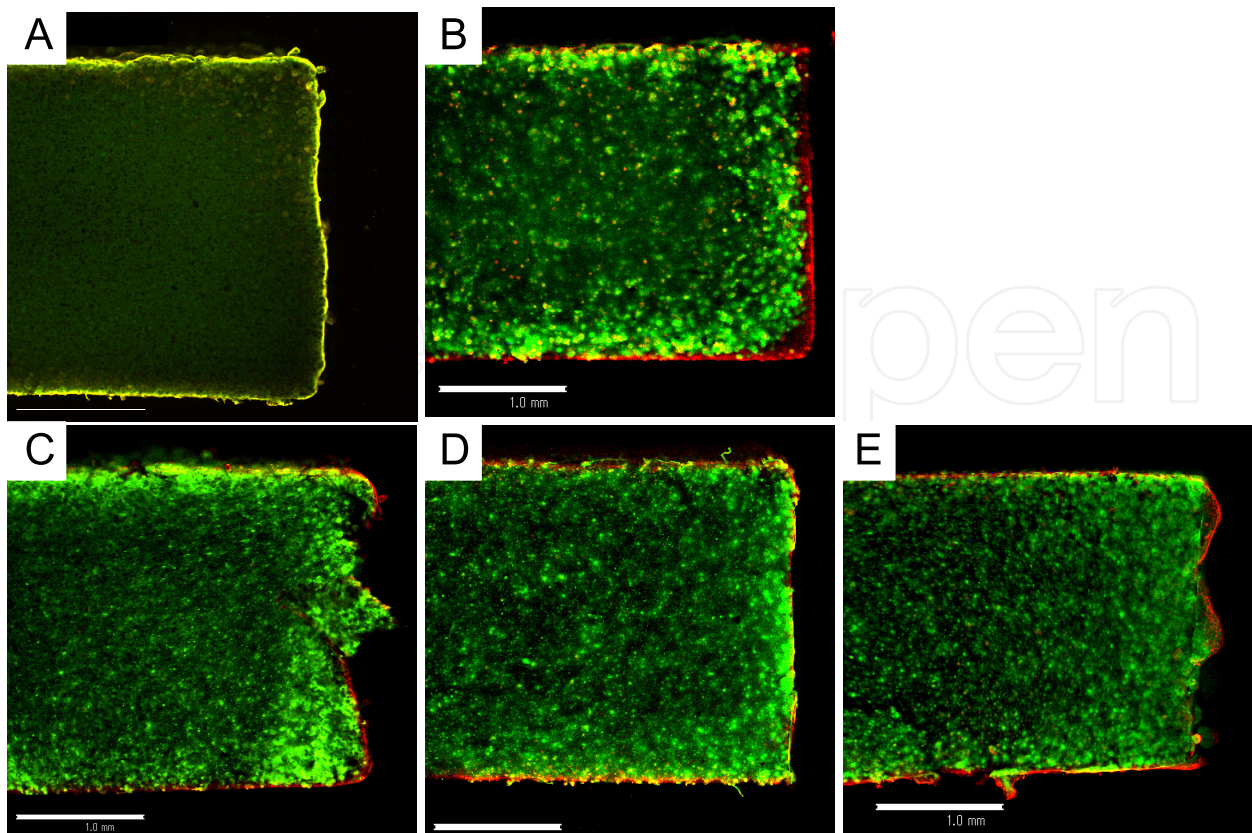
Collagen synthesis is enhanced by increasing VC concentration [23, 24, 28, 29]. In addition, the immunofluorescent images in Fig. 5 indicate that the synthesized collagen fibrils, as the chief reinforcing fiber in the elaborated tissue, are upregulated by increasing AsA concentration. Moreover, the self-assembled collagen network was spread out spatially in the construct and interconnected among chondrocytes. As shown in Fig. 5, the elaborated ECM is forms into a large network which spreads infinitely. This means that entanglements between opposite sides of a cube in three-dimension exist, and that the entire construct is percolated by interconnecting the ECM network, as shown in Fig. 9. Therefore, the mechanical property of the developed ECM network appeared as that of the whole construct.



**Figure 6.** Comparison of the tangent modulus after 22 day culture period. Cultures were terminated following either a free-swelling (white column), control (gray column) or compression (black column) condition. Each number in a column is cultured sample number and each sample represents an individual culture. Error bar means *SD*. Sharps (# and ##) and asterisks (\* and \*\*) were shown statistical significant respective as compared to control group and between each groups in Welch's *t*-test. #, \*:  $d > 1, P < 0.01, (1 - \beta) > 0.8$ ; ##, \*\*:  $d > 2, P < 0.01, (1 - \beta) > 0.8$ .

The observed mechanical behavior of the cultured constructs, in which the elastic modulus changed non-linearly with the culture period, was similar to the mechanical characteristics of the gel at the vicinity of the sol-gel transition point [30]. In particular, the elasticity of the gel in the vicinity of the sol-gel transition point is exponentially proportional to the molecular concentration of polymer solution [31]. It was widely alleged that the gel had been formed by the very tenuous network irrespective of the mechanism of cross-linking formation process and had been dominated by entropic elasticity in the vicinity of the gelation point Several studied several researches with experiments [31, 32] and numerical simulations [33–35].

On the basis of the above, the chondrocytes synthesize collagen fibrils around themselves, and reinforced polymer concentration ( $\rho$ ) in the cultured construct is enhanced by increasing collagen density. Then, the elasticity was increased exponentially by culture period ( $t$ ) as



**Figure 7.** Low-magnified immunofluorescence images of ECMs under either free-swelling (A, B, C), control (D) or compression (E) culture condition: A: without VC; B: AsA(2.2); C–E: A2P(6.4). Blue: type I collagen; green: type II collagen; red: chondroitin sulphate. Culture period was 22 days. Each trichrome stained samples represents an individual culture. Scale bar represents 1 mm.

follow,

$$E \propto |\rho - \rho_c|^\lambda \propto t^\nu, \quad (1)$$

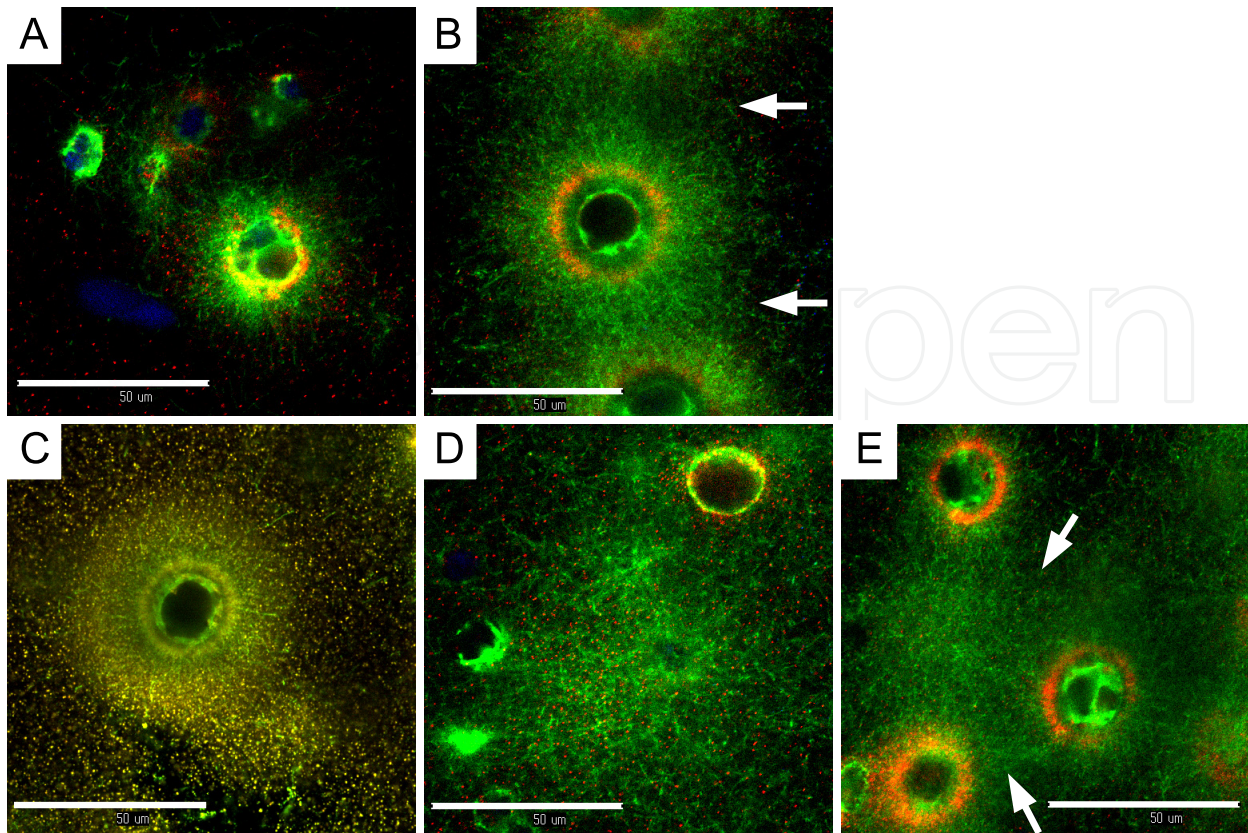
where  $\rho_c$  is polymer density at gelation point. We considered that the elastic modulus of the regenerated cartilage is expected to exponentially proportionate with the culture period. Therefore, we hypothesised that the tangent modulus of cultured construct increases exponentially with the culture period because the ECM content of the cultured constructs improved with the culture period by the following relationship,

$$\frac{E}{E_0} \propto t^\nu, \quad (2)$$

where  $E_0$  is tangent modulus at day 1 of the culture period and exponent  $\nu$  is the growth rate of the tangent modulus. To investigate the relationship between AsA concentration and the growth rate of the tangent modulus, we applied equation (2) to the tangent moduli of all experimental groups in Fig. 4 and described these exponents ( $\nu$ ) into Fig. 10. As shown in Fig. 10, the exponent ( $\nu$ ) was linearly proportional to the AsA concentration with  $3.2 \text{ pmol}/10^9$  cells as follows,

$$\nu \propto (\text{AsA concentration}). \quad (3)$$



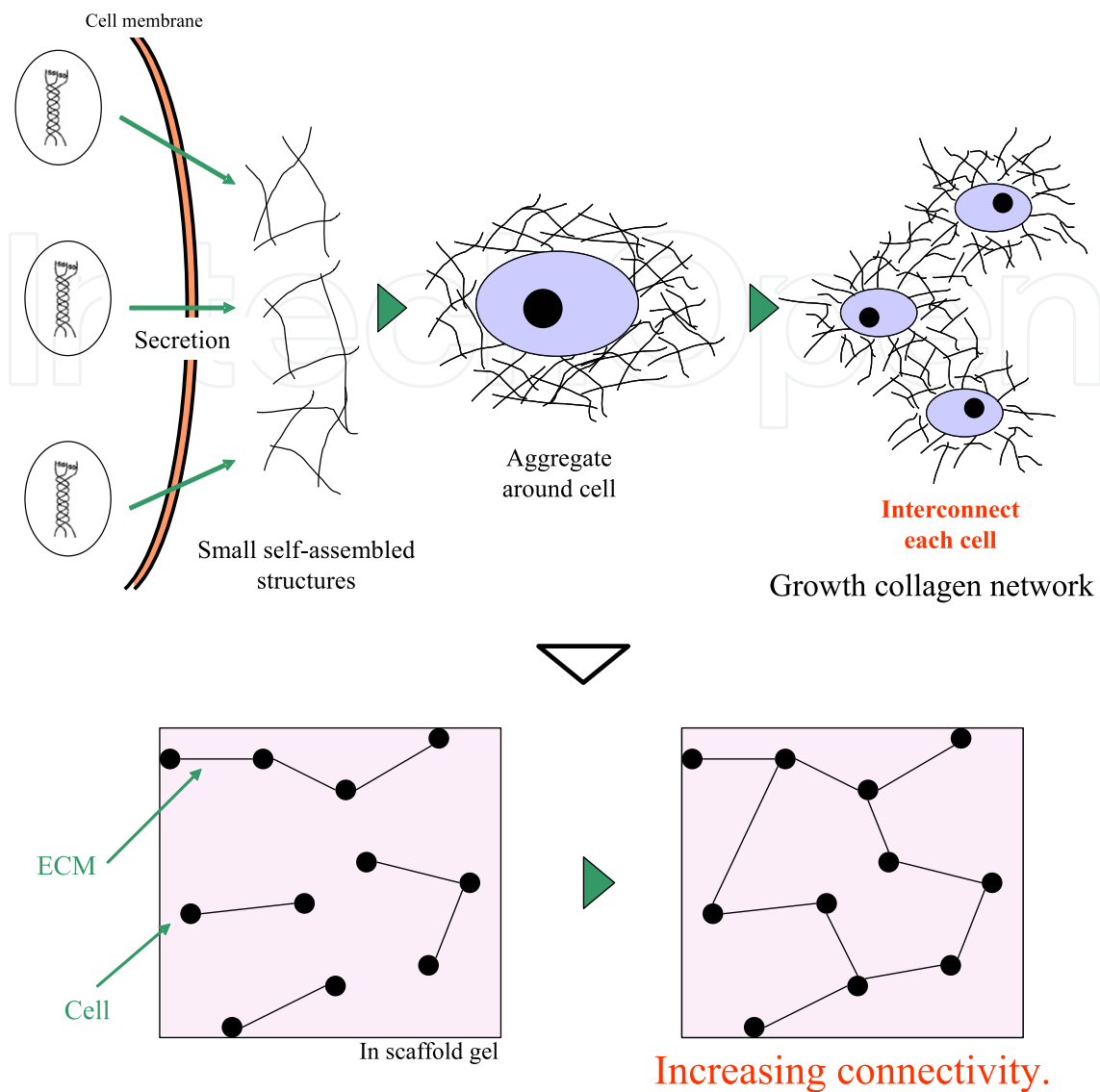


**Figure 8.** High-magnified immunofluorescence images of ECMs in the vicinity of the tissue model under either free swelling (A, B, C), control (D) or compression (E) culture condition: A: without VC; B: AsA(2.2); C–E: A2P(6.4). Blue: type I collagen; green: type II collagen; red: chondroitin sulphate. Culture period was 22 days. Each trichrome stained samples represents an individual culture. Scale bar represents 50  $\mu\text{m}$ . Arrows indicate to interconnect among chondrocytes by type II collagen net work.

To establish a suitable design method for regenerating tissue-engineered cartilage, we studied the effect of two types of chemical and physical stimulation on the mechanical properties of the chondrocyte-agarose construct as a regenerated cartilage model. One is to add a high quantity of VC to the culture medium to effectively enhance collagen synthesis. The other is to apply a compressive strain to the construct using a purpose-built bioreactor. Our results showed that the compressive strain led to an increase in the tangent modulus because it was clear that the collagen network had increased its density and had interconnected chondrocytes.

#### 4.2. Influence of mechanical stimulation on the development of the ECM and mechanical properties

To establish a suitable design method for regenerating tissue-engineered cartilage, we studied the effect of two types of chemical and physical stimulations on the mechanical property of the chondrocyte-agarose construct, as a regenerated-cartilage tissue model. One is to add high VC concentration into culture medium for effectively enhancing the collagen synthesis. The other is to apply a compressive strain to the construct using a purpose-built bioreactor. Our results showed that compressive strain had led to increase tangent modulus because it

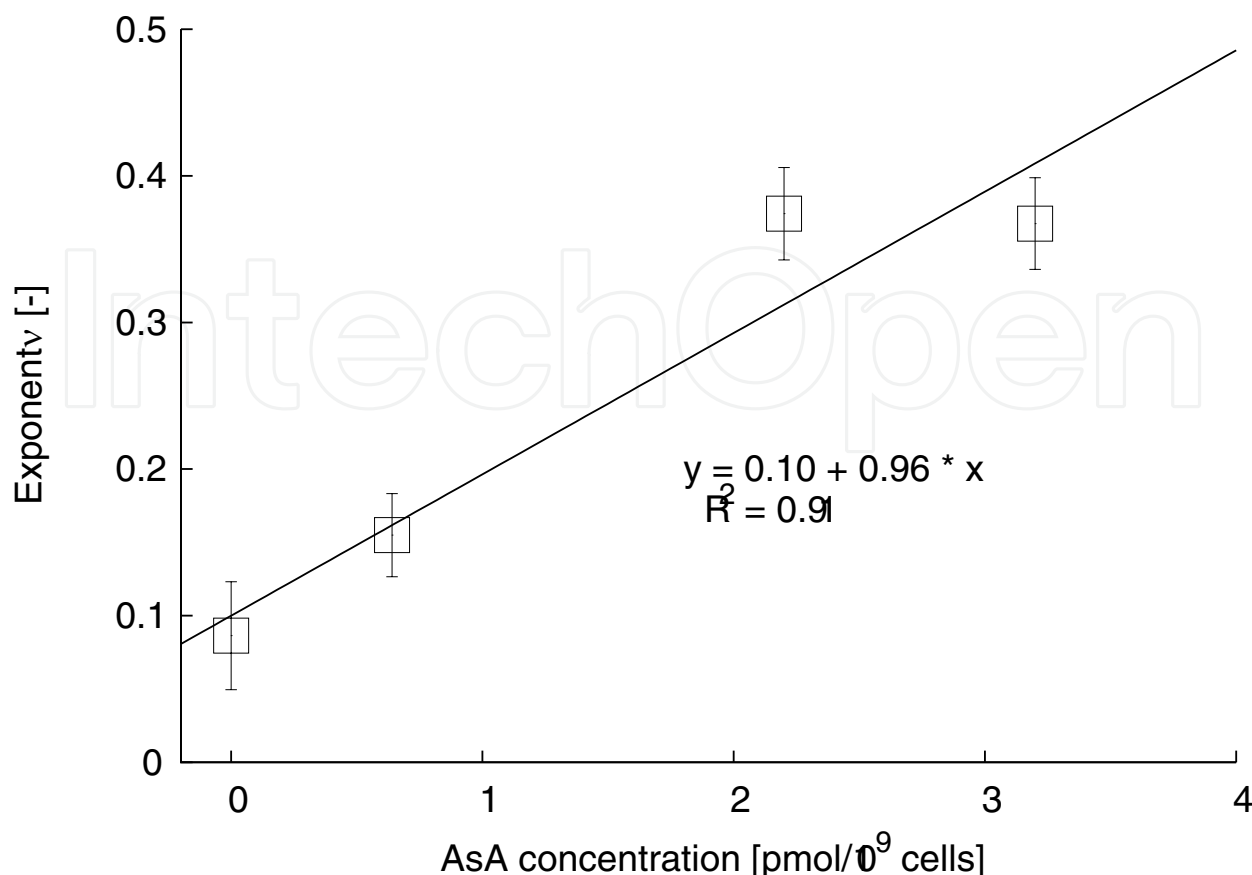


**Figure 9.** Developing process of ECM and increasing connectivity. Elaborated ECM forms large network which is spreaded infinitely. This means a entanglement between opposite sides of a cube in three dimensions, and that entire mechanical property was percolated by of interconnected ECM network.

had been clear that collagen network had become dense and had been interconnected among chondrocytes. We already described in last section (4.1) that it had definitely important that chondrocytes had been linked by ECM for development of the elasticity of the construct.

It is well-known that A2P has no physiological activity but can produce the same as that of effect of VC activity after dephosphorylation by an alkaline phosphatase (ALP) [36]. Dephosphorylated A2P, this is AsA, penetrates into chondrocyte through a VC transporter, chiefly the sodium-dependent VC transporter 2 (SVCT2), and supports the collagen synthesis as a cofactor in the rough endoplasmic reticulum [37]. Under free-swelling culture conditions, we observed few collagen molecules distributed in the construct and found that the tangent modulus of the A2P group was lower than that of the AsA(2.2) group. We should also consider reaction rates, one of A2P dephosphorylation by ALP, the other of AsA transport into the





**Figure 10.** Relationship of AsA concentration and exponent. Line shows a linear regression in AsA concentration interval of 0 to 3.2 pmol/10<sup>9</sup> cells.  $R$  is the coefficient of determination.

cytosol by SVCT2. The Michaelis constants of both ALP and SVCT2 of bovine chondrocyte is 1–10 and  $62 \pm 25 \mu\text{M}$  [38, 39]. Since the affinity of the substrate for ALP is slightly higher than for SVCT2, AsA concentration around chondrocyte in A2P group was lower unlike cultivation of high AsA concentration, the also rate of collagen synthesis was relatively decreased by comparison with AsA dose group. Then, the tangent modulus of the free-swelling groups with the A2P was suppressed with restraining cytotoxicity of AsA.

When applying compressive strain to the construct, the tangent modulus of A2P(6.4) was higher than that of the respective free-swelling groups. This is because the collagen fibers interconnected chondrocytes and because the collagen network of these groups was expanded. We think this mechanical stimulation enhanced the diffusion of both A2P and nutrients, homogenizing A2P in the construct. In addition, the stimulation probably excited a mechanosensor on the cell surface, activating cell-signaling pathways, namely the mechanotransduction pathway. It is well-known that mechanical stimulation causes chondrocytes to several biological responses in cartilage remodeling strategies which are based on the implantation of a cultured tissue [40]; for example, activation of the mitogen-activated protein kinase (MAPK) pathway, a kind of cell signaling pathway [41, 42], increase in GAG biosynthesis [43] and regulation of inflammatory species synthesis [44]. These chondrocyte responses were followed presently after loading mechanical stimulation and sustained the activity of MAPK pathway for 5–60 min [41, 42]. BY synthesizing ECM with mechanical stimulation, chondrocytes built up the collagen network and adapted to changes

in the deformation around the cells. Therefore, the tangent modulus of the compression group with A2P was higher than that of the free-swelling group.

Homogenously developing ECM under a free-swelling culture condition for expansive regeneration of cartilage is a complex process. Our results provide evidence that it is necessary to subject the construct to mechanical strain because nutrients should be supplied uniformly to the construct. We also think that a diffusion of nutrients exists under free-swelling culture conditions. The diffusion coefficient of water in agarose gel is about  $10 \times 10^{-10} \text{m}^2\text{s}$  [45, 46]. This means that a water molecule diffuses 10 mm per a day in the gel when applying the Fick's law. Pluen *et al.* reported that the diffusion coefficients of the proteins, lactalbumin and ovalbumin, in 0.1 M PBS(-) solution into agarose gel was  $0.8\text{--}1 \times 10^{-10} \text{m}^2\text{s}$  [47]. This means that the nutrient particles of diffuse 3 mm per a day in the gel. Moreover, if the ECM of the cultured construct is more densely synthesized, it is hard to diffuse nutrients in the tissue; and consequently, the diffusion coefficient will be decreased. Thus, if a medical doctor would like to implant a large quantity of regenerated cartilage to treat the cartilage defect, the construct should be subjected to mechanical stimulation to homogenously develop the ECM network.

## 5. Summary

This chapter demonstrates the relationship between the development of the ECM in the construct and the mechanical properties of the construct for establishing a suitable design method to reconstruct the regenerated cartilage by using a chondrocyte-agarose construct as a tissue model. First, we revealed the influence of VC concentration in the culture medium on the mechanical properties of the regenerated cartilage model. The present findings suggest that the mechanical characteristic of the construct depend clearly on the AsA concentration in the culture medium. The tangent modulus of the cultured construct was exponentially increased according to cultivation duration. The growth rate of the tangent modulus was accelerated to upregulate ECM secretion in the high AsA concentration. This study strongly suggests, therefore, that the mechanical properties of the regenerated cartilage depend on the interconnections created by the ECM between cells which are mainly those of the three-dimensional collagen network. Second, we investigated the influence of mechanical properties on the development of the ECM network as well as the regulation of the chondrocyte-agarose constructs using two types of VCs, AsA and A2P. Neither the collagen network nor the tangent modulus of the A2P dose groups was improved compared with the AsA group under the free-swelling culture condition. Moreover, it is clear that the free-swelling culture condition suppresses the development of the ECM of the inner tissue compared with the ECM of outer tissue. When applying compressive strain to the construct, the tangent modulus of the A2P dose group was increased because the ECM networks of the inner tissue had been upregulated and had interconnected chondrocytes. We can additionally consider that mechanical stimulation enhanced the diffusion of nutrients and improved the synthesis of the ECM via the mechanotransduction pathway. Moreover, we revealed that it is necessary to apply mechanical stimulation to a large engineered tissue when treating articular cartilage defects.

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