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Measurement of diffusion in articular cartilage using fluorescence correlation spectroscopy

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Abstract

Background: Fluorescence correlation spectroscopy (FCS) provides information about translational diffusion of fluorescent molecules in tiny detection volumes at the single-molecule level. In normal states, cartilage tissue lacks vascularity, so chondrocyte metabolism depends on diffusion for molecular exchanges. The abundant extracellular matrix (ECM) of cartilage is maintained by a limited number of chondrocytes. ECM plays an important role in the regulation of chondrocyte functions. In this study, FCS was used to measure diffusion behaviors of albumin, the major protein of the intra-articular space, using normal and degenerated cartilage. Preliminary investigation of fluorescence dyes including Alexa 488, Rhodamine 6G and Rhodamine 123 was conducted to evaluate their properties in cartilage.

Results: The results indicate that the diffusion behaviors of fluorescently labeled albumin can be observed using FCS in both normal and chemically degenerated cartilage.

Conclusions: This work demonstrates the capability of FCS for direct measurement of diffusion in cartilaginous ECM. When the diffusion characteristics of fluorescent probes in ECM are clarified using FCS evaluation, FCS will be applicable as a method for early diagnosis of osteoarthritis, which is accompanied by increased abnormalities of ECM and also as tool for evaluating bio-engineered artificial cartilage for autologous chondrocyte implantation.

Background

Fluorescence correlation spectroscopy (FCS) is a highly sensitive method based on analysis of fluctuations in fluorescence intensity to detect and characterize fluorophores in living cells as well as in solution. For instance, FCS allows real-time measurement of two important physical parameters for biochemistry: the average number of molecules in the detection space; and the translational diffusion constant of the molecules through the open volume of detection [1-4].

Cartilage tissue is an avascular tissue, and allows the exchange and transport of nutrients, gases, and metabolites by continuous diffusion instead of through the vasculature [5]. Diffusion in extracellular matrix (ECM) of normal cartilage is thus central to the physiobiological nature of chondrocytes. Cartilage tissue principally consists of ECM and a small number of chondrocytes. The abundant ECM in cartilage is secreted by these chondrocytes. Although ECM provides an environment for the molecular exchanges needed for chondrocyte survival, and plays an important role in physiological activities for the regulation of chondrocyte function, intimate communications between cells and alterations of metabolism, almost no studies have examined the diffusion behaviors of particular molecules from synovial fluid through the ECM of cartilage. Some studies have examined diffusion characteristics and diffusion across articular cartilage using dyes [6,7], glucose [6] and hydrogen [8], To investigate the normal pattern of every different type of molecules in ECM of cartilage, large-scale experiments and varying samples are required. These efforts may help define intricate phenomenon of diffusion in cartiliginous tissue.

Since synovial fluid makes a significant contribution to the nutrition of articular cartilage with direct movement of particular molecules from the synovial space to cartilage by diffusion, understanding the diffusion patterns of molecules from synovial fluid is important. Pathological changes to the ECM cause osteoarthritis (OA), altering not only the physical metabolism of chondrocytes, but also normal molecular exchanges in cartilage.

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In this study, FCS analysis is made to evaluate albumin movements by means of diffusion in cartilage. Since albumin is the major protein of synovial fluid, tracing albumin protein movements may reveal differences between normal and abnormal states of cartilage.

The purpose of this study was to evaluate the feasibility of FCS for diffusional analysis in normal and chemically degenerated cartilage in relation to albumin, as a representative protein. To select a suitable fluorescent dye before application to cartilage tissue, the physical parameters of several fluorescent dyes were tested and documented. We chose to use a model of degenerated cartilage created by chemical treatment for FCS evaluation, to reflect the denaturation of ECM that might be expected in cartilage tissue during OA.

Results

Degeneration Model of Articular Cartilage

Figure 1 shows the histological appearance of trypsin-treated cartilage samples. As demonstrated by the staining results for porcine articular cartilage using HE, toluidine blue and safranin O, normal cartilage tissue changed into degenerated tissue over time and staining patterns altered after enzyme treatment. At the starting point of enzyme digestion (0 h), cartilage samples showed uniform staining throughout with toluidine blue and safranin O. However, increasing degeneration resulted in larger loss of such staining over time, showing extensive loss of proteogylcans in the tissue.

After 7 h of digestion, no staining was detected in samples using toluidine blue or safranin O, with only HE staining remaining. Therefore, treating cartilage plugs for >7 h was considered to achieve suitable models of degenerated cartilage in these experiments. When 6 h had passed, center regions of digested samples still showed a small portion of intensive staining with safranin O and metachromatic staining with toluidine blue, demonstrating that normal ECM constituents are still present, unlike samples treated for >7 h in which ECM protein components have totally disappeared. Degenerated cartilage and untreated normal cartilage samples were used in FCS measurements.

FCS Measurements

Diffusional behaviors (diffusion coefficient) for all fluorescent dyes utilized in this research were detected by FCS monitoring in PBS solvent. Optimal concentrations of fluorescent dyes differed, which may have resulted from the different chemical, properties of dyes in solusions. The optimal concentrations of Rhodamine 123, Rhodamine 6G, Alexa Fluor 488 hydrazide, and Alexa Fluor 488 conjugated with albumin form bovine serum were, 10^{-7}M, 10^{-7}M, 10^{-8}M, 10^{-5}M, respectively. Two different HA, Artz (MW; 8.0 $\times 10^5$, Seikagaku, Tokyo, Japan) and Suvenly (MW; 2.0 $\times 10^6$, Chugai Pharmaceutical, Tokyo,

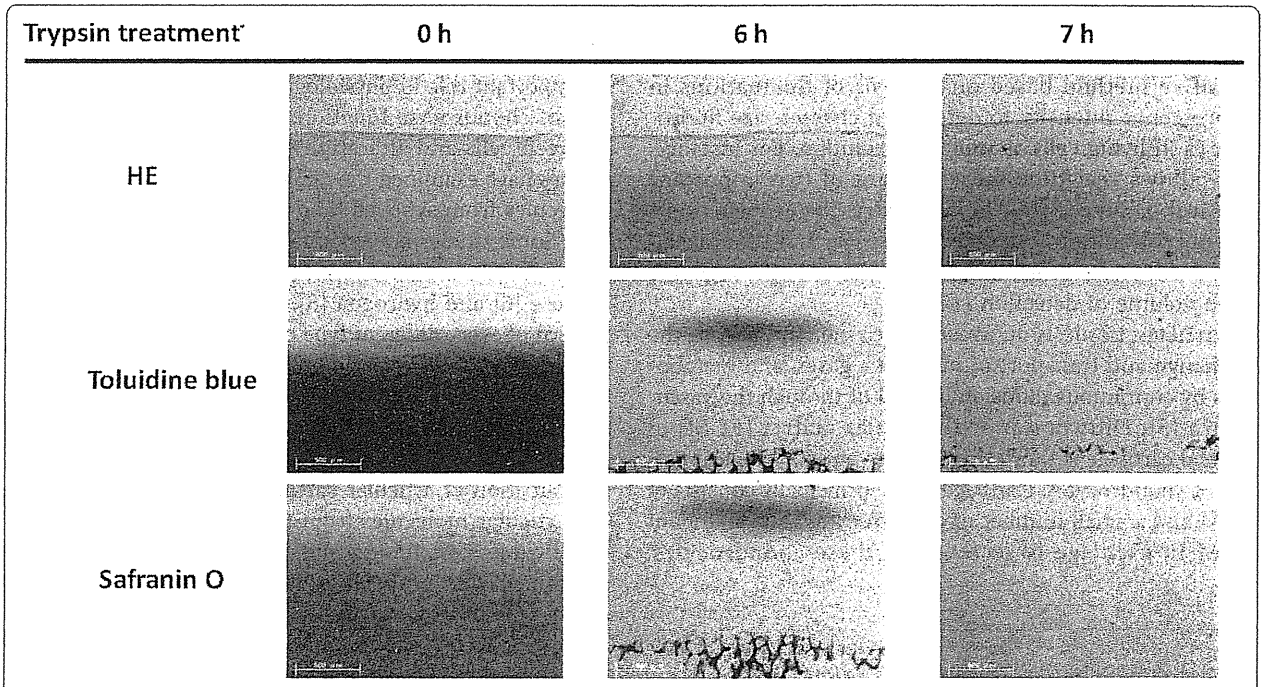

Figure 1 Results of histological examination. After treatment for 7 h, no staining was detected in samples with toluidine blue and safranin O stains, but only HE staining. Therefore, cartilage plugs treated for >7 h were considered as degenerated cartilage models in these experiments. Scale bar = 500 μm.

Japan) were used and their concentrations were 0.1 wt%. Diffusion coefficients of the fluorescent dyes were in the order of PBS > HA (MW, 8.0 $\times 10^5$) > HA (MW, 2.0 $\times 10^6$) (Figure. 2).

Changes of diffusion coefficients are related to the MW of dyes and apparent viscosity of aqueous solution. Fluorescent dyes with a larger MW showed lower diffusion coefficients. At the same time, diffusion coefficients of probe dyes in solution containing HAs decreased with increasing MW of HAs.

The fluorescent dye validated in HAs and cartilage tissues was accepted as a FCS probe, and then applied in the next tests. Since the maximum measurable depth for FCS equipment using Alexa dyes was greater than that using Rhodamine dyes (data not shown), Alexa Fluor 488 was selected as the FCS probe.

FCS measurement tests were performed with Alexa Fluor 488 labled-albumin to trace the diffusion motion of albumin in both normal and degenerated cartilage. FCS data demonstrated an intimate correlation between measureable points (depths from the superficial surface) and enzyme-treatment times (Figure. 3). Increments in these points were correlated with prolongation of trypsin treatment times. FCS data were validated at the range of 120 μm when digestion was conducted for 9 h and >9 h, showing that FCS monitored permeation of the FCS probe at this depth. These focus distances were defined as maximum measurable depths (MMD). When the focus moved over these ranges, no correlation curves were formed, indicating that no movement and no localization of fluorophores is detected in the testing field. As a result of MMD detection, an MMD of 20 μm was chosen for cartilage tissue in the present study.

FCS measurement data for Alexa Fluor 488 conjugated with albumin from bovine serum (Alexa-albumin) as the FCS probe are summarized in Figure 4, which

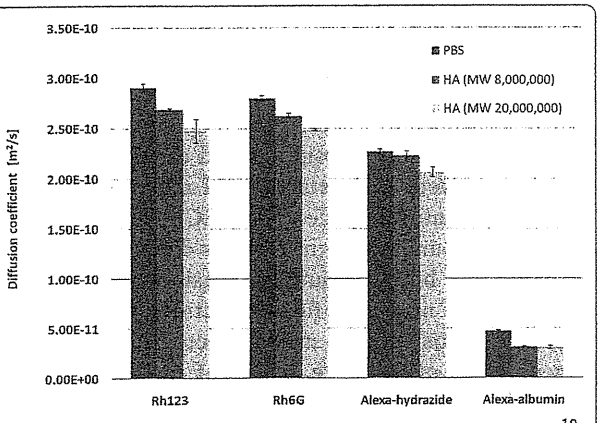

Figure 2 Diffusion coefficients of fluorescent dyes, E-10 = $\times 10^{-10}$. Diffusion coefficients of fluorescent dyes were in the order of PBS > HA (MW, 8.0 $\times 10^5$) > HA (MW, 2.0 $\times 10^6$).

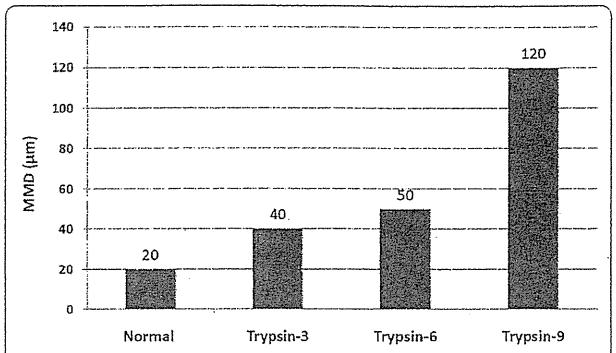

Figure 3 Maximum measurable depth (MMD). FCS data demonstrated an intimate correlation between measureable points, MMD, and prolonging the reaction time of trypsin treatment.

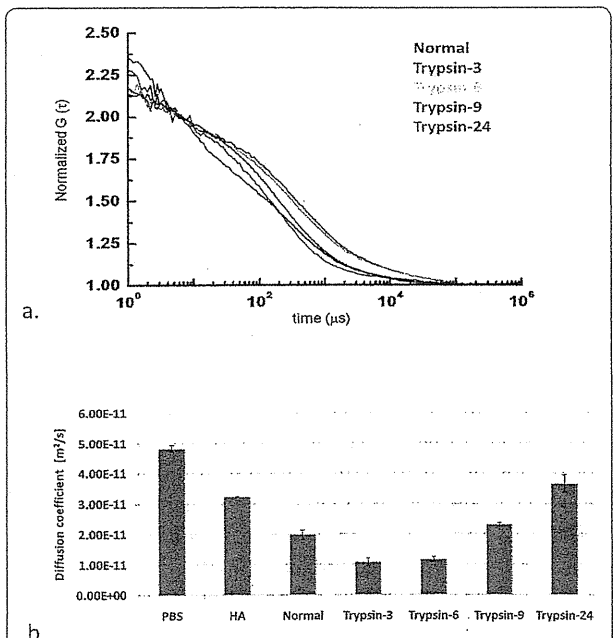

Figure 4 Summary of the FCS measurements. a. Representative of auto correlation curves for Normal, Trypsin-3, Trypsin-6, Trypsin-9, and trypsin-24 b. Diffusion coefficients of Alexa-albumin, E-10 = $\times 10^{-10}$. FCS data demonstrated that diffusion coefficients of Alexa Fluor 488 conjugated with albumin (Alexa-albumin) were greater when measuring degenerated cartilage, indicating that FCS probes moved more freely in degenerated cartilage than in normal tissue. Interestingly, diffusion coefficients for trypsin-3 and trypsin-6 were lower than in normal cartilage. p values of each of groups were under 0.01 which demonstrated the significance of the difference between the groups excluding three of p values between the groups; HA vs.Trypsin-24, Normal vs. Trypsin-9, and Trypsin-3 vs. Trypsin-6. Among these higher values over 0.01, p value between No significant difference was found between Trypsin-3 and Trypsin-6(p = 0.486). Besides, we found a significant difference in the rest of two values which were below 0.05 (p = 0.042; between HA and Trypsin-24, and p = 0.021; Normal and Trypsin-9).

explains the diffusion behaviors of albumin under varying circumstances. We selected one HA with an MW of 8.0 $\times 10^5$ as representative and performed analyses for this HA. Diffusion coefficients of Alexa-albumin in PBS, HA, trypsin-treated cartilage for 3 h (Trypsin-3), trypsin-treated cartilage for 6 h (Trypsin-6), trypsin-treated cartilage for 9 h (Trypsin-9), and trypsin-treated cartilage for 24 h (Trypsin-24), were 4.83 $\times 10^{-11}$, 3.23 $\times 10^{-11}$, 1.06 $\times 10^{-11}$, 1.15 $\times 10^{-11}$, 2.30 $\times 10^{-11}$, and 3.64 $\times 10^{-11}$ m^2/s, respectively. In addition, non-treated normal cartilage was 1.97 $\times 10^{-11}$, between the ranges of Trypsin-6 and Trypsin-9. An increase in diffusion coefficients was seen with increased duration of chemical digestion.

p values of each of groups were under 0.01 which demonstrated the significance of the difference between the groups excluding three of *p* values between the groups; HA vs.Trypsin-24, Normal vs. Trypsin-9, and Trypsin-3 vs. Trypsin-6.

Discussion

FCS is an extremely sensitive method for providing concentration and diffusion constant, and molecular interaction of fluorescent molecules in a small volume (femtoliters) of complex mixtures [1-3]. The major sources of fluctuations within the confocal volume are molecular diffusion by Brownian motion, convection, and chemical reactions that change the fluorescence yield. The parameters of molecular dynamics can then be easily extracted by analyzing the time correlation function of fluorescence fluctuations. Information from FCS monitoring is expressed by the autocorrelation curve, then diffusion behaviors are analyzed for comparison. Recent research has been extensively applying FCS measurements to material transportation and interrelationships among biomolecules at the level of the single living cell, and even at the ultra-micro level of intracellular spaces in small organelles [3,9,10].

One important cartilage proteoglycan is HA (comprising glucuronic acid and N-acetyl-glucosamine). This molecule is one of the major components in synovial fluid. HA molecules are also present in cartilage matrix as the backbone structure in proteoglycan aggregates. Since HA plays a major role as an organizer of the ECM [11], we selected HA as a test solution for a FCS probe of cartilage tissue in addition to PBS as a solvent.

In the present study, we tried to measure diffusion properties in cartilage tissues using FCS methods. Before this step, a suitable FCS probe was needed to trace the motion of albumin, and preliminary investigation of several fluorescence dyes was conducted to assess their properties. All diffusion characters of the fluorescent dyes in the present research were detected by FCS equipment in the solvent of PBS and HAs. Since rhodamine dyes showed unstable when applied to cartilage tissue, Alexa dyes utilized as FCS probe. This instability may concern several reasons of character of rhodamine itself such as its strong absorption, tendency to dimerize at higher concentration[12] as so on. Further investigation is needed to determine most proper dye when fluorescent dye is applied to other tissue.

OA is caused by alterations in proteoglycans, degeneration of the collagen network, and an increase in fluid content [13]. Experimental treatments using specific enzymes such as trypsin can simulate these changes [14,15]. In this study, we chose cartilage degeneration created using short-term trypsin treatment as a model of OA. Trypsin treatment of tissue caused a marked loss of proteoglycans in the cartilagenous tissue (Figure. 1). Degenerated cartilage was defined with treatment for >7 h, showing no staining with toluidine blue or safranin O. This result is consistent with previous findings [15].

FCS data demonstrated that diffusion coefficients of Alexa-albumin were greater when measuring degenerated cartilage (Figure. 4), indicating that FCS probes moved more freely in degenerated cartilage than in normal tissue. This phenomenon may occur due to the gradual destruction of ECM structures, finally giving probes a chance to move in a wider space than that in normal cartilage. Diffusion coefficients provide diffusion characteristics of certain molecules. Molecules with larger MW showed lower diffusion coefficients (Figure. 2). Diffusivity decrease with increasing molecular size of flurorescent dyes. Simultaneously, specific molecule described by the diffusion coefficient is affected by the solution environment as shown by tests of HAs showing decreasing diffusion coefficients with increasing MW. Therefore, lower diffusion coefficients indicate difficulties for molecules to diffuse within the surrounding conditions, matching the present results. We were therefore satisfied that the diffusion coefficient for Alexa-albumin can explain the diffusion characteristics in PBS, HAs and cartilage tissue. Interestingly, diffusion coefficients for trypsin-3 and trypsin-6 were lower than in normal cartilage. Considering parameters of diffusion equations and our findings from this study together, this result may be explained as follows. When an aggressive chemically digestion using trypsin induced cartilage degeneration is initiated, early-stage ECM denaturation may change the natural space of the ECM to much more intricate surroundings with exudates digested ECM constituents that hinder albumin diffusion and even have possibility of non-specific interaction with albumin. Moreover, this finding may reflect the time-related degeneration of cartilage, which of early stage differs from that of late stage. Once cartilage has been treated for >6 h, albumin is more diffusible and thus shows an increased diffusion coefficient compared to normal cartilage.

Synovial fluid is a lubricant of intra-articular surfaces and a source of nutrients for hyaline cartilage by diffusion. Synovial protein concentration averages around 42% of the concentration in serum [16]. Among these proteins, albumin constitutes the single largest protein fraction (55-86% of total synovial protein) [16], representing a major contribution to the role of colloid osmotic pressure and other physiobiological functions. As a result, understanding the diffusion characteristics of albumin in such synovial fluid is valuable. Alexa-albumin is made by adding labeled albumin from bovine serum to Alexa-hydrazide. The diffusion behavior of this fluorescent dye was reproducible and simulated experimentally with an *in vitro* model tracing the movement of albumin. Alexa-albumin happened to be commercially available, but FCS probes using other molecules will be also feasible as candidates to create a standard for understanding physiological diffusion in the living body. In addition to albumin as a target molecule for FCS probes, further studies regarding new FCS molecular probes will be essential in the future. The present results reveal that the diffusion state of fluorescence-dyed albumin can be determined by FCS measurement regardless of the intensity of cartilage degeneration.

Some studies have already examined diffusion of particular dyes in cartilage tissue [6-8]. However, methods in those experiments were unsuitable for application as diagnostic tools. These studies needed large volumes of cartilage tissue and were based on macroscopic (gross) experimental data, representing an extremely high level of invasiveness. One of the experiments with dyes and glucose was utilized to evaluate the mechanisms of diffusion across the cartilaginous membrane in a two-compartment device [6]. To diagnosis the pathological alterations in cartilage clinically using diffusion information, simple preparation of samples for examination is critical for achieving minimally invasive diagnosis. In a similar trial in terms of using fluorescence materials and tiny detection fields, Hardingham et al. developed sensitive methods for assessing the matrix assembly around chondrocytes, based on the use of confocal fluorescence recovery after photobleaching (confocal-FRAP) to determine the translational diffusion of fluorescent tracer molecules of defined size [17]. However, this technology was not devised to measure diffusion of particular molecules directly in the ECM, but rather to elucidate the conditions for matrix assembly itself.

More recently, studies have been reported and they showed different methods of measuring diffusion in cartilage using different fluorescence techniques [18-28]. Histological evaluations were conducted on tidemark and calcified cartilage of histological sections with fluorescence agents (fluorecein and rhodamine) [24] itself, and fatty acid labeled with rhodamine fluorescence and albumin[25] using quantitative fluoresce microscopy.

Several researchers reported photobleaching methods pioneered with fluorescence recovery after photobleaching (FLAP) [18,26], fluorescence loss induced by photobleaching (FLIP) [19] and scanning microphotolysis (SCAMP) [20-23]. Fluorecein [18,19], fluorescently-labeled dextran (FITC-conjugated dextran) [20-23] and fluorecein-conjugated bovine serum albumin [26] were utilized as a fluorescent materials to evaluate the diffusivity of human annulus fibrosus [18], calcified cartilage of deep region [19], pericellular matrix of porcine articular cartilage [21], cartilage in normal state [20,23,27,28] or during compression with mechanical stress [22,27,28], ligament [23], growth plate [28] and even agarose [26] and tissue-engineered cartilage [20]. These studies have reported the diffusive transport properties of solutes in both cartilage and collagenous tissue. Some of the experimental results for diffusion coefficients, determined by various methods using fluorescent probes, are summarized in Table 1. According to this table, diffusion coefficient (D) ranged from 2.0×10^{-14} to 290.0×10^{-10} m^2/s. Nonetheless, most of numbers for diffusion coefficients measured with different fluorescent dyes are of similar magnitude to those that measured with FCS in our experiment, on the order of 10^{-11} to 10^{-10}. Our adaptation of the FCS technique can be used to measure site-specific diffusivity in an extremely small detection volume of tissue. The diffusion coefficients measured with Alexa-albumin in the normal cartilage (1.97×10^{-11} m^2/s) and degenerated cartilage (1.06×10^{-11}, 1.15×10^{-11}, 2.30×10^{-11}, and 3.64×10^{-11} m^2/s), are in good agreement with values of the order measured previously using other techniques (3.1×10^{-11} m^2/s using fluorescence recovery after photobleaching and 4.0×10^{-11} m^2/s using radiotracertracking [21]. Among these previous studies with tagged with albumin probes [25,26], the diffusion coefficients in cartilage ranged from 0.3×10^{-11} to 29.0×10^{-11} m^2/s (Table 1). Considering the range of methods and possible variation in the properties of various cartilage sources, present results (Figure. 4) are in reasonable agreement with these data, supporting the accuracy of our methods. Advantages of our methods include quickness of diffusion measurements, simplicity, noninvasiveness, and the ability to quantify the molecular diffusion in the different individual tissues.

The apparently wide range of diffusivities of normal cartilage and degenerated cartilage highlights the influence of physical properties of both the fluorescent molecule and the ECM on hindered transport within biological systems. This may results from the circumstantial and collateral conditions such as tissue conditions (animal species, type of cartilage, preservation until measurement, compression etc.), solutions utilized

Table 1 Summary of experimental results for diffusion coefficient, *D*, from recent studies using fluorescent dye

Fluorescent dye	Method	Specimen	Temp. (°C)	D ($\times 10^{-10} m^2/s$)	Ref.
Fluorecein (332 Da)	Fluorescence recovery after photobleaching (FRAP)	Human intervertebral discs	22	0.38 ± 0.25 ~ 2.68 ± 0.84	[18]
		Inner, middle and outer regions of annulus fibrosus			
Fluorecein (376 Da)	Fluorescence loss induced by photobleaching (FLIP).	Murine (C57BL6J) distal humurs	4		[19]
		Subchondral bone		0.0002 ~ 0.012 (0.0007 ± 0.0003)	
		Calcified cartilage		0.0005 ~ 0.009 (0.0026 ± 0.0022)	
Fluorescein isothiocyante (FITC)-tagged dextran (3, 40, 70, and 500 kDa)	Fluorescence recovery after photobleaching (FRAP)	Tissue engineered cartilage from human adipose-derived stem cell with or without scaffold (alginate, agarose, fibrin and gelatin)	37	0.16 ± 0.08 (Day 28, cultured within fibrin in control media using 500 kDa)	[20]
			or	~ 18.10 ± 3.94 (Day 1, cultured within gelatine in chondrogenic media using 3 kDa)	
			?		
Fluorescein isothiocyante (FITC)-tagged dextran (70 kDa)	Scanning microphotolysis (SCAMP).	Porcine femoral condyle	?		[21]
		Healthy cartilage			
		Extracellular matrix		0.23 ± 0.02	
		Pericellular matrix		0.19 ± 0.02	
		Osteoarthritic cartilage			
		Extracellular matrix		0.23 ± 0.02	
		Pericellular matrix		0.23 ± 0.02	
Fluorescein isothiocyante (FITC)-tagged dextran (70 kDa)	Scanning microphotolysis (SCAMP) and Fluorescence imaging of continuous point photobleaching (FICOPP)	Porcine femoral condyle	?		[22]
		Normal cartilage		0.33	
		Compressed cartilage		0.07	
Fluorescein isothiocyante (FITC)-tagged dextran (3 and 500 kDa)	Fluorescence imaging of continuous point photobleaching (FICOPP)	Collagenous tissues	4	Inexpressible because authors explain diffusivity by not diffusion coefficient but by diffusivity ratio for comparisons	[23]
		3% agarose gel	or		
		Lateral collateral ligaments (Porcine)	?		
Rhodamine B (443 Da, cationic), Rhodamine B (479 Da, neutral but polar), Fluorecein (332 Da), and Na-fluorecein (376 Da)	Quantitative fluorescence microscopy on histological sections	Equine forelimb	4	0.0098 ± 0.0013 ~ 0.037 ± 0.003	[24]
		Subchondral bone			
		Calcified cartilage			
Bovine serum albumin labeled with rhodamine - maleimide and Nitrobenz -2-oxa-1,3-diazole (NBD) -labelled lauric acid (378 Da) bound to the fluorescent albumin	Quantitative fluorescence microscopy on histological sections	Equine metacarpal-phalangeal joints	4	9.0 ± 2.0 (48 h-incubation, using albumin)	[25]
				~	
				290.0 ± 10.0 (2 h-incubation, using lauric acid)	

Table 1 Summary of experimental results for diffusion coefficient, *D*, from recent studies using fluorescent dye *(Continued)*

Fluorescein-conjugated bovine serum albumin (66 kDa).	Fluorescence recovery after photobleaching (FRAP)	3~8% agarose gel	24	0.164 ± 0.018 ~ 0.411 ± 0.008	[26]
		Porcine growth plate		0.0387 ~ 0.4922	
Tetramethylrhodamine (TMR) -tagged dextran (3,10, and 40 kDa) and tetramethylrhodamine (430 Da) itself,	Novel experimental apparatus and desorption fluorescence method.	Bovine femurs	4	0.19 ± 0.02 (8% compression, using 40 kDa dextran)	[27,28]
		Compressed cartilage		~ 0.52 ± 0.06 (8% compression, using 430 Da TMR)	

For studies investigating anisotropic diffusivity, the smallest to the largest value of the diffusion coefficient is reported. (Temp.: Temperature, Ref.: Reference).

(ingredient, ion contents, buffer, culture media, manufacturing company etc.), tissue processing (treatment and incubation time, condition, time, temperature etc.), and properties of each fluorescent dye (shape, molecular weight, physical properties electric charge, the hydrophilic or hydrophobic natures, manufacturing company ect).

Diffusion coefficients can also be measured by fluorescence recovery after photobleaching (FRAP). However, most of these methods to analyze FRAP data expect the homogeneity in the measurable field of the bleached area and fail to assume geometrical restrictions to diffusion. Accordingly, diffusion coefficients in inhomogeneous materials, such as most biological tissues, cannot be evaluated correctly.

Several methods are available to analyze FRAP data, each with its own characteristics [29]. The technique to apply depends on the data which are aimed for and the tissue that is being probed. Presumably the most adaptable tool currently utilized is by spatial Fourier analysis of a sequence of FRAP images [30]. With this method, anisotropic diffusion, flow, matrix binding, and diffusivity in multiple components of a gel can be evaluated, whereas the evaluation is independent on the geometry of the bleached area [26]. This limitation originates from the requirement that the boundary of the image must have a constant intensity value. In practice, this means that a large area, relative to the bleached area, is to be imaged. This decreases the amount of signal in the images. The same requirement of constant boundary intensity applies to this method [26]. The average intensity of the images is allowed to change during the measurements. In practice, this means that the bleached area typically constitutes a large part of the acquired images to enhance the signal. Note that the lower limit to the physical size of the bleached area is defined by the point-spread function. This needs to be considered if small bleached areas are used [26].

Compared with photobleaching methods, FCS need minimum excitation power. Hence, this technique requires lower power and much small amount of fluorescent dyes to get information of FCS data. It is not easy to calculate directly the diffusion coefficient with photobleaching tools, however, we can promptly get the absolute value of diffusion coefficient with FCS instrument. One of the merits using FCS is that various concentrations of the fluorescent molecules used are easily monitored. It is worth considering the complementary use of FCS and photobleaching methods with their different characteristics.

To the best of our knowledge, no previous studies have demonstrated the feasibility of FCS for direct measurement of diffusional behaviors of fluorescently labeled albumin in the ECM of cartilage tissue, and this approach may represent a potential and useful evaluation tool. If diffusion in the ECM can be clarified and categorized with this new method by standardization of FCS data under various cartilage conditions, FCS will be applicable for the early diagnosis of OA, which is accompanied by increased destruction of ECM elements, and also as a tool for evaluating bio-engineered artificial cartilage for autologous chondrocyte implantation. Besides changes in diffusion characteristics of molecules in the cartilage ECM, additional and complementary information can be adopted to clarify the clinical picture. For example, alterations in the ECM simultaneously induce changes in viscoelasticity of the cartilage. Monitoring changes in viscoelasticity is possible using reliable techniques such as photoacoustic measurement [15,31,32]. Such information could be used together with diffusion characteristics to evaluate optimal conditions for ECM and to test bio-engineered neocartilage constructs, and will suggest new criteria for real-time evaluation with small quantities of samples under minimally invasive arthroscopic surgery with FCS analysis system.

Conclusions

This work offers the first demonstration of the capabilities of FCS for direct measurement of diffusion behaviors of ECM in cartilage. This sensitive measurement

technique provides great advantages in detecting diffusible molecules due to the ability to achieve rapid measurements from small sample volumes.

Methods

Sample Preparation

Fresh swine knees (n = 5) were obtained from a local slaughterhouse at Kanagawa meat center (Frieden, Kanagawa, Japan). Cartilage tissue from the femur was prepared within 4 h as follows. The cartilage tissues of delivered knee joints were cut out into cylindrical cartilage plugs (diameter, 5 mm; depth, 1 mm; n = 240) using a biopsy punch (Kai Industries, Seki City, Japan) and disposable scalpels (Akiyama, Tokyo, Japan). Porcine cartilage specimens were initially incubated under physiological conditions (37°C, 5% CO_2) in physiological saline (Otsuka Pharmaceutical Factory, Tokushima, Japan) until the next procedure.

Degenerated Cartilage Models

Addition of enzymes was used for experimental degradation of the tissue matrix using phosphate-buffered saline (PBS) (Wako Pure Chemical, Osaka, Japan) containing 0.1% trypsin solution (1 mg/ml; Invitrogen, Carlsbad, CA, USA) to degrade primarily proteoglycans. The trypsin treatment time was minimally 1 h and varied up to 24 h every hour to control the extent of degeneration.

To stop the trypsin reaction, an equal volume of fetal bovine serum (FBS) (Invitrogen) was added and then incubated for a further 30 min. The digested cartilage samples were thoroughly rinsed with PBS to remove residual trypsin and FBS.

Chemically treated and non-treated cartilage discs were then divided for histopathological assessment and FCS measurement. To perform the histopathological assessment, samples were fixed in 4% paraformaldehyde and embedded in paraffin, and 4-mm-thick sections were prepared. Histological staining was performed using hematoxylin and eosin (HE), toluidine blue and safranin O to visualize the degree of ECM degeneration within specimens. Samples for FSC were prepared and used for FCS measurements with the FCS probes as described below.

Preparation and Selection of Fluorescent Dye

To determine properly applicable fluorescent dyes (FCS probes) for cartilage tissue, commercially available fluorescent dyes were obtained, including Rhodamine 123 (Rh123) (molecular weight (MW), 380.82; Sigma-Aldrich, St. Louis, MO) Rhodamine 6G (Rh6G) (MW, 479.01; Sigma-Aldrich), Alexa Fluor 488 hydrazide (Alexa-hydrazide) (MW, 570.48; Molecular Probes, Eugene, OR). First, optimal concentrations of the fluorescent dyes in PBS (100 μl) were measured and analyzed, then applicable concentrations of each dye solution (100 μl) were mixed with 100 μl of hyaluronic acid sodium (HA) and tested.

To optimalize the concentrations of these fluorescent probes, FCS measurement were conducted with ten-fold serial dilutions of each fluorescent agents adding PBS. After receiving the FCS data from these measurements, the calculatable data of each concentration of dyes were obtained by expressing autocorrelation curve.

Two different molecular sizes of HA with average MWs of 8.0 $\times 10^5$ (Artz; Seikagaku, Tokyo, Japan) and 2.00 $\times 10^6$ (Suvenly; Chugai Pharmaceutical, Tokyo, Japan) were used in this experiment.

In addition to these measurements, albumin-conjugated fluorescent dyes were adopted to evaluate the diffusion behavior of albumin protein. Alexa Fluor 488 conjugated with albumin from bovine serum (Alexa-albumin) (MW approximately 66,000; Molecular Probes) was tested and analyzed with PBS, two differ types of HA solutions and cartilage samples. Physiological parameters affecting the diffusion behaviors of fluorescence dyes were measured and monitored with FSC measurement, including counts per molecule, count rate, diffusion time, particle number, correlation, structure parameter, triplet fraction and triplet time. In the case of measurement within cartilage tissues, length from the superficial surface to the maximum measureable points was experimentally determined.

Statistical Analysis

All results of the experiments are expressed as the means ± SE. The mean values for each group were compared by ANOVA and then by using Fisher's least significant difference method. Values of $p < 0.05$ were considered the minimum level of statistical significance

FCS Measurement and Analysis

FCS was performed using an LSM510-ConfoCor 2 system (Carl Zeiss, Oberkochen, Germany), as described elsewhere [33,34]. FCS measurements of all samples were recorded at 25°C.

Various concentrations of the candidate fluorescent dyes were incubated with purified PBS at 37°C in an atmosphere of 5% CO_2 and 95% air over 30 min. Aliquots (100 μl) were arrayed onto Lab-Tek chambered cover-glass (Nalge Nunc International, Naperville, IL, USA) with eight wells and <140-μm-thick cover-glass on the bottom. Cartilage specimens were placed on Lab-Tek chambered cover-glass with eight wells and 100 μl of FCS probe-solution was applied over the samples.

Each acquired correlation data set was analyzed by software supplied by Carl Zeiss with a fitting program (FCS Access Fit software; EVOTEC BioSystems, Hamburg, Germany), or exported to Igor Pro software (IGOR Pro 5.05a; Wavemetrics, Lake Oswego, OR). In the FCS analysis, the diffusion coefficient is represented by the average of five FCS measurements

The autocorrelation curve is obtained by correlating the fluorescence intensity trace shifting within a time interval. The time shift τ is varied, and the correlation curve is obtained by multiplying the deviation of the average intensity, δF, at the time point t with the deviation at time point $t + \tau$ and averaging over the whole trace. Finally, the correlation function, $G(\tau)$, is normalized with the squared average signal.

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F \rangle^2} \quad (1)$$

Further practical considerations in the calculation of FCS curves from a fluorescence intensity trace are detailed elsewhere [35-37].

Diffusion of one single component is commonly fitted with the standard model [38]:

$$G(\tau) = 1 + \frac{1}{N}\left(1 + \frac{4D\tau}{{\omega_0}^2}\right)^{-1}\left(1 + \frac{4D\tau}{{z_0}^2}\right)^{-\frac{1}{2}} \quad (2)$$

The resulting ideal probe volume is approximated by a Gaussian profile with the extension ω_0 in x and y directions and z_0 in the z direction [39]. N is the number of fluorescence molecules in the detection volume, defined by a radius ω_0 and a length 2 z_0. The diffusion time (τ_D) is related to the traditional diffusion constant of the diffusion coefficient D. As this time corresponds to Equation 3, the diffusion coefficient D is obtained:

$$\tau_D = \frac{{\omega_0}^2}{4D} \quad (3)$$

The diffusion of spherical molecules is related to various physical parameters by the Stokes-Einstain equation as follows:

$$D = \frac{k_B T}{6\pi\eta r} \quad (4)$$

where T is the absolute temperature, r is the radius of the spherical molecule, η is the fluid-phase viscosity of the solvent, and k_B is the Boltzman constant.

When measuring the diffusion time of samples (τ_{sample}) and rhodamine 6G (τ_{Rh6G}) with the FSC system, the diffusion coefficient of rhodamine 6G at 20°C [40], 2.8 $\times 10^{-10}$ m^2/s, was used as an authentic value for determination of the diffusion coefficient of samples (D_{sample}) measured on the expectation of a proportional relationship based on the following equation:

$$\frac{D_{sample}}{D_{Rh6G}} = \frac{\tau_{Rh6G}}{\tau_{sample}} \quad (5)$$

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Authors' contributions
JIL and KU performed the research. JIL, KU and MS analyzed the data. JIL took charge of the statistical analyses. JIL, KU, MS, and JM wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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関節軟骨損傷修復のための軟骨細胞シート

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背景

われわれは，これまでに関節軟骨の修復·再生に関して，基礎的研究を主に *in vivo* 実験で確認してきた。例えば，組織工学的手法による軟骨再生に適した担体作製に関する研究[1]，至適細胞外環境の構築に関する研究[2)~5)]，同種の組織工学的軟骨，線維輪移植による組織修復·再生に関する研究[6)~9)]，ならびに軟骨細胞シート移植による軟骨修復·再生に関する研究[10)11)14)~16)]などである。これら一連の研究により，軟骨の修復·再生におけるホスト(レシピエント)由来細胞とドナー由来細胞との相互作用の重要性を確認した。そして，組織修復·再生に必要最小限のイニシエーター(組織工学的軟骨)があれば，ホスト由来細胞が主導的に組織修復を促進することを見いだした[6)13)14)]。

温度応答性培養皿による軟骨細胞シート

温度応答性培養皿は，東京女子医科大学岡野光夫教授が開発したもので，独自のナノ表面設計により温度応答性ポリマー(PIPAAm)を器材表面に固定化することで器材表面は32℃を境に可逆的に疎水性(細胞接着表面)親水性(細胞遊離表面)に変化する。この特性により，トリプシン等，細胞に損傷を与える酵素を一切用いることなく，温度を20~25℃にして10~30分程度待つだけで，無傷な細胞と細胞外マトリックスがシート状に回収可能である。温度応答性培養皿で作製した積層化軟骨細胞シートは，通常の培養皿で得られる培養細胞とは異なる特性を有し，これらを利用した関節軟骨修復再生効果をわれわれは初めて報告し[10)]，組織修復能力に優れた積層化軟骨細胞シートの特性を明らかにした[11)15)16)](図1)。

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軟骨細胞シートによる関節治療の可能性

軟骨細胞シートは力学的には脆弱ではあるが，優れた接着性を有し，損傷した軟骨からのプロテオグリカンの流出を阻止し，関節液中のカタボリックファクターから軟骨を保護し，成長因子の持続的な供給源であると共に，さらに骨髄由来幹細胞の軟骨分化を促進するイニシエーターとしても機能しており，単なる軟骨再生というよりは，むしろ自己修復能力を向上させる効果により，軟骨は修復·再生されている。つまり，変形性関節症において常に混在しながら存在する軟骨全層欠損(軟骨下骨まで達する骨軟骨損傷で，従来の再生医療のターゲット＝図2A)と軟骨部分損傷(図2B)の両タイプの軟骨損傷に対して，細胞シートによる修復·再生効果を確認し，細胞シート工学という日本発のオリジナルな技術により，変形性関節症の治療にまで踏み込んだ再生医療の実現を目指している。

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図1 細胞シート工学を応用した関節軟骨修復・再生

われわれは温度応答性培養皿を用いて，酵素を用いないで非侵襲的に整形外科領域の種々の細胞をシート状に回収し，さらに積層化にも成功している。細胞シートは，優れた接着性とバリア機能を有し，損傷部からのプロテオグリカン流出を阻止し，関節液中の破壊因子から損傷部を保護し，成長因子の持続的供給源としても機能するため，良好な修復再生効果が期待できる。

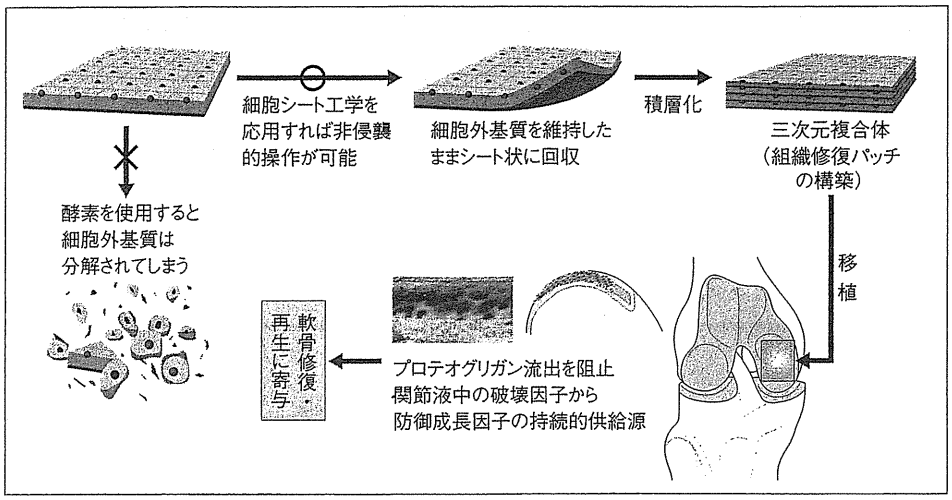
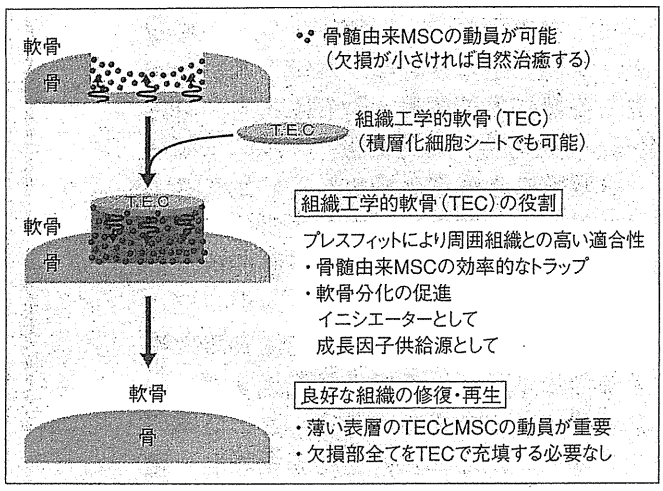
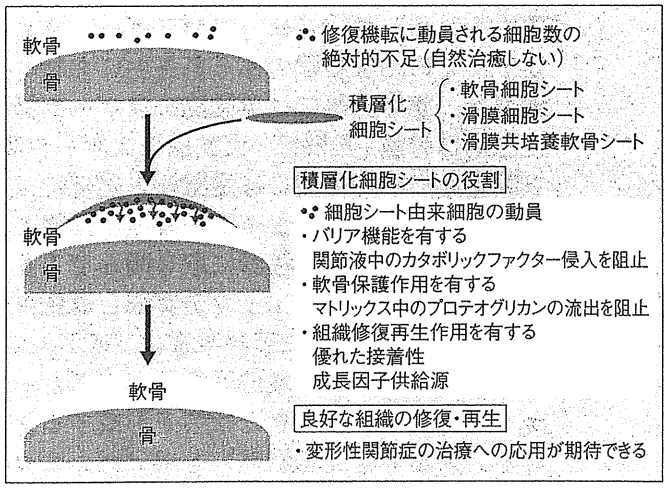


図2 関節軟骨損傷の修復・再生機序

A 関節軟骨全層欠損の修復・再生機序。軟骨全層欠損の場合，表層部分のみを組織工学的に作製した薄い軟骨で置換さえすれば，良好な修復・再生が生じることを動物実験で確認し，報告してきた。そして，移植する組織工学的軟骨をさらに薄くして行って，3層の積層化軟骨細胞シートでも軟骨再生が可能であることを，ウサギとミニブタの動物実験で確認した。

B 関節軟骨部分損傷の修復・再生機序。軟骨部分損傷に治療効果があることが確認されたのは，世界で初めてであり，変形性関節症のように2種類の軟骨損傷，すなわち軟骨全層欠損と部分損傷が常に混在する疾患に対する治療効果が期待できる。





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関節軟骨修復・再生を目指した軟骨滑膜混合細胞体の開発

李　禎翼[1,2]　佐藤正人[1]　三谷玄弥[1]
持田譲治[1]

抄録：自家軟骨細胞移植法は骨軟骨損傷の治療に臨床応用されているが，高齢者の広範囲病変には適応困難であることや体外培養期間が長いなど，解決すべき点がある．日本白色家兎を用いて採集量に制限のある軟骨細胞に高度な増殖能と軟骨細胞への分化能を保有する滑膜細胞を加えたスフェロイド細胞移植体を新規に作製した．本法により短期間のうちに多量の移植体の準備が可能となり，臨床へのより効率的な応用が期待できる．

＊ The development of cellular spheroids using synovial cells and chondrocytes for the autologous chondrocyte implantation

Key words：autologous chondrocyte implantation, ACI 自家軟骨細胞移植法，synovial cells 滑膜細胞，cellular spheroids 細胞スフェロイド

はじめに

1994年Brittbergらが発表した[1]自家軟骨細胞移植（autologous chondrocyte implantation, ACI）法は，すでに全世界で2万例以上の骨軟骨損傷患者に臨床応用された実績がある．しかし，患者の正常軟骨部から採取できる移植用の関節軟骨細胞の採集量には制限があり，広範囲の病変は適用外で，さらに培養による準備期間が約4週間と長いなど，なお改善すべき点がある．

また，高齢者の場合，再生能が乏しい軟骨細胞（AC）は培養増殖能が低く，広範囲な病変の治療は困難であった．一方，高い増殖能をもつ滑膜由来細胞（SY）には間葉系幹細胞（以下MSC）の存在と軟骨細胞への分化能が報告されている[3]．

今回，われわれは，浮遊状態でACとSYからなる混合細胞を振とう培養する新たな培養法を開発し，多量の細胞移植体を短期間で準備することを可能とした．本研究の目的は，この軟骨滑膜混合細胞移植体の性状を経時的に観察，分析し，新規ACI法としての応用可能性を明らかにすることである．

材料および方法

本研究は東海大学医学部動物実験倫理委員会の承認を得て行った．

1．軟骨細胞滑膜由来細胞の分離・培養

ウサギ（n＝4，Japanese White Rabbit，1 kg±

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200 g，雌）膝関節から採取した細胞の培養はMitaniらの方法[8]に準じて行った．ウサギの膝から軟骨組織および滑膜組織を採集し，細切した組織は1.25%トリプシン（Invitrogen Co.）および，0.5%コラゲナーゼI（collagenase class I：Worthington, Biochemical Co.）溶液により2段階消化して単離した．各細胞は，DMEM/F-12培地（Gibco. Co.）で非動化した10%のFetal Bovine Serum（FBS，HyClone），1%の抗菌剤/抗真菌剤混合物（Anti-Anti：Invitrogen Co.）および50 μg/mlのアスコルビン酸（ビタミンC注10%PB，Nissin Pharma Inc.）で調製された増殖用培地を用いてpolystyrene制培養皿（表面面積；500 cm^2，Corning）にて各細胞は2.0×10^4 /cm^2の密度で播種した．培地交換は週2回行い，90%のコンフルエントに達した段階で継代培養を実施した．各継代過程において，SYとACの培養日数あたり継代増殖率を算出した［(回収時細胞数/播種時細胞数)/培養日数)］．統計学的検討は，Student's t検定を用いてP<0.05を有意差ありとして検定した．

2．蛍光試薬による標識および細胞スフェロイドの作製

蛍光顕微鏡にて経時的にスフェロイドの形態を追跡するためPKH染色キット（Sigma Co.）を用いて，ACは赤色蛍光（MINI26），SYは緑色蛍光（MINI67）で蛍光標識した．

その後，増殖用培地中でACとSYの含有条件を変えて，細胞数の和が6.0×10^6/5 mlとなるように調製した（図1a～c）．

直径60 mmの細胞非接着性培養皿（Hydro-Cell™，CellSeed Co.）に各細胞懸濁液を入れて，振とう培養用シェーカー（モデル名：ダブルシェーカーNR-3，TAITEK Co.）にて平面円形の流動（70 rpm）を与えながら振とう培養によるスフェロイド培養を行い，37℃，5%CO_2の条件下のインキュベーターの中で3～5日間（125時間）培養を維持した．

3．細胞スフェロイドの性状分析

各条件の細胞材料懸濁液からなる細胞スフェロイドの形成過程を，蛍光顕微鏡（IX-70型倒立顕微鏡，Olympus）を用いて経時的に観察した．

細胞スフェロイドは，4%のパラホルムアルデヒド溶液とスクロース溶液に浸漬した後，凍結切片とした．作製した切片はhematoxylin and eosin（H & E）染色，toluidine blue染色およびsafranin O染色を行い，さらに，Collagen type 1, 2に対する抗体（Anti-hCL（I），Anti-hCL（II），Daiichi Fine Chemical Co.）とABC（avidin-biotin peroxidase complex，DAKO Japan Co.）法による酵素抗体法にて免疫組織化学的染色を行って鏡検した．

分子生物学的な検討（RT-PCR）として，検査資料（n=4）として上記の各条件の細胞材料懸濁液（スフェロイド培養開始時のday 0）と3日間スフェロイド培養して回収した細胞スフェロイド（day 3）を，Dnase digestionとspin column purification過程を含むSV Total RNA Isolation System kit（Promega）を用いてRNAを抽出した．

各primer（図2b）を用いて抽出した各RNAはTaqManRT reagents（Applied Biosystems）とrandom-hexamerによってcDNA化した．内在性のコントロール（internal standard）として18S ribosomal RNA（Applied Biosystems）を用いた．TaqMan universal PCR master Mix（Applied Biosystems）とABI SDS7300（40 cycles under standard thermal conditions；Applied Biosystems）によって反応させ，day 0とday 3において各遺伝子のRNAレベルの発現を3% NuSieve 3：1アガーロスゲル（Lonza）にて電気泳動し，ethidium brimideで染色した後，densitograph system（ATTO Biotechnologies）によって，各増幅バンドを撮影した．

結　果

1．培養日数当たり継代増殖率

ウサギ1膝からACは平均850.92×10^4個，SYは平均593.32×10^4個が回収された．

12～13日間の細胞増殖の継代培養期間中にACは2回，SYは最大4回まで継代ができ，細胞スフェロイドの作製には，第3継代目のACと第5継代目のSYを用いた．培養日数当たり平均継代増殖率は，ACは0.51±0.11，SYは0.96±0.29であり，有意にSYで高かった（図2a）．

2．細胞スフェロイドの作製および分析

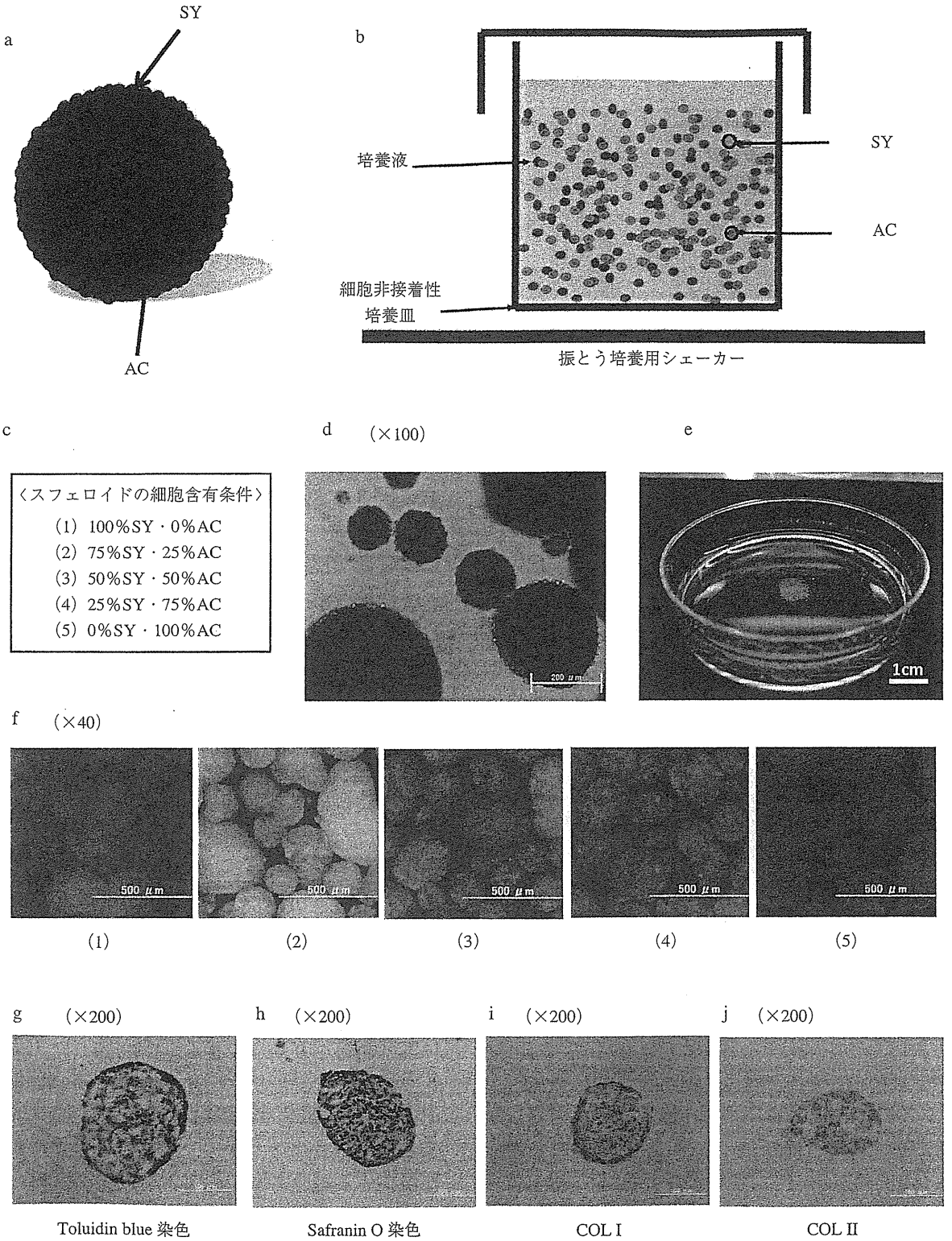


図1 細胞スフェロイド

a, b：細胞スフェロイドおよびスフェロイド培養の模式図（SY：滑膜由来細胞，AC：軟骨細胞），
c：スフェロイドの細胞含有条件，
d：細胞スフェロイド作製後の顕微鏡下観察像，
e：肉眼的な観察所見，
f：各細胞スフェロイドの蛍光顕微鏡下観察像，
g～j：胞含有の条件75%SY・25%ACで作製した細胞スフェロイドの組織学的検討

ACおよびSYは，振とう運動による持続的な反復により培養容器の底部にとどまらず，培養液中に浮く懸濁状態で維持でき，振とう初期段階に現れた小さいサイズの混合細胞塊を中心として多数の細胞同士が迅速に接着するようになり，経時的に細胞スフェロイドが成長した．5つの細胞含有条件のすべてにおいて細胞スフェロイドの作製が可能であった（図1d～f）．混合細胞塊は，スフェロイド培養開始12時間後から肉眼的にも観察可能であった．

振とう培養開始125時間後には細胞スフェロイドは凝縮して輪郭が滑らかになった．細胞スフェロイドのサイズは，小さいものは直径が250±100 μm，大きいものは700±250 μm程度であった（図1d, f）．

図1g～jに，細胞含有条件を75%SY・25%ACとして作製した細胞スフェロイドの染色像を代表例として示すが，細胞含有条件とtoluidine blue染色による異染性の程度との関連性は認められず，いずれも局所的な異染性を示した（図1g）．Safranin O染色の結果も同様にすべての細胞スフェロイドの内部では全体的な染色陽性を認めず，局所的に乏しい染色性を示した（図1h）．

Collagen type 1, 2に対する免疫組織化学的染色の結果（図1i, j）では，両者の細胞スフェロイド内部にて部分的な発現が認められた．

各条件の細胞スフェロイドを対象に実施したmRNAレベルのCollagen type 1, 2の発現は，すべての細胞含有条件において確認できた（図2b）．

考　察

本研究では，ACIへ利用可能な新規移植体を作製するために，採集量に制限があるACと同時にSYを採択して混合細胞スフェロイドを形成し用いた．現況ではACIにACとSYがともに移植体として研究され利用するようになりつつあり，AC[10]，SY[13]を用いるACIの研究では，細胞単独の移植のさまざまな不利な点が明らかになり，最近では体外培養時に各細胞と組織再生用基材のスキャフォールドを利用して移植体を構築した後移植する方法が主流である[2,6]．特にSYは，個体年齢と関係なく優れた増殖能を表し，MSCの局在も確認されている．さらに，軟骨細胞への容易な分化能から最近多く研究されるようになってきた[3]．しかし，現在までACとSYを混合した細胞移植体の例はなく，われわれの本研究が初めての報告である．

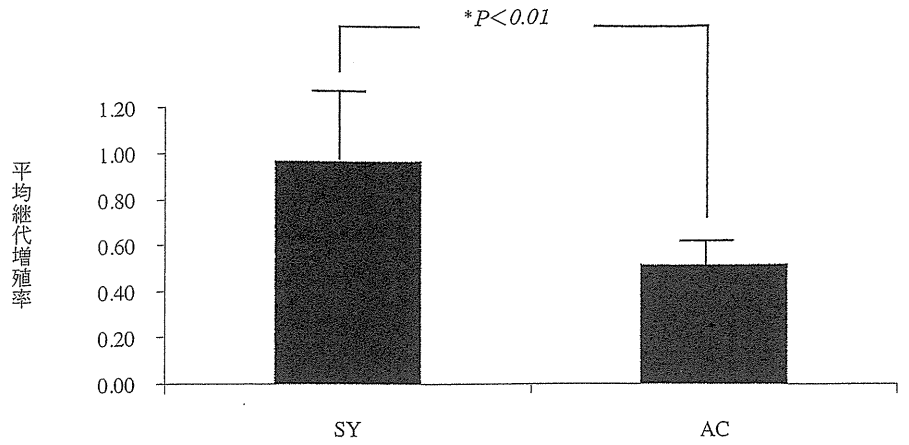
種類の異なる細胞同士が混在する構造物を構築することは困難であるが[4]，本研究の作製法ではスフェロイド培養初期では細胞同士の接触の機会が多く細胞同士の塊を形成しやすくなり，振とう培養から起きる培養液の流動は，細胞スフェロイドの構造をより緻密にする．この人為的に与える力によって，混合細胞の移植体の作製にいたった．

培養日数当たりSY，ACの平均継代増殖率は，およそ2倍程度SYが有意に高く，12日間の継代培養期間中，理論的にSYが1024（2^{10}）倍多い増殖量が獲得可能である．実際の細胞スフェロイド作製に用いた継代培養で増幅されたSY，ACは，初代培養時の播種した細胞より，それぞれ50～100倍，10～20倍の増殖が可能であった．

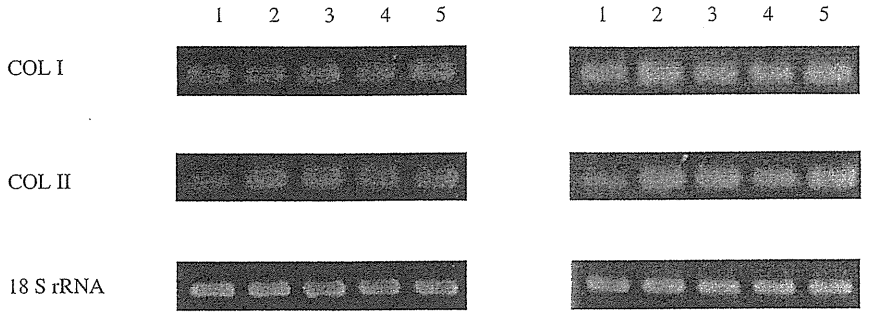
形態学的および組織学的な検討の結果，toluidine blue染色では異染性は弱く，safranin O染色性も乏しかった結果とCollagen type 1, 2の免疫組織化学的染色の結果，発現には多少程度の差はあったものの，すべての細胞スフェロイドにおいて確認できたことから考えると，移植前の細胞スフェロイドは，体外の *in vitro* の環境でのスフェロイド培養の期間が短時間であったため，構成する各細胞は完全に軟骨細胞および軟骨様組織として形質発現していないものと推測できる．これと同様に軟骨組織の分化度を示すCollagen type 1, 2に対する分子生物学的検討の結果，短期間の培養期間中の混合細胞塊の性状は経時的にもその様態もさまざまであったことから，分化と脱分化した細胞が混在する細胞スフェロイドの内部の状況の軟骨分化程度を評価することは困難であった．

一方，われわれは今までも組織工学的手法による軟骨再生に適した担体作製に関する研究[7]，至適細胞外環境の構築に関する研究[12]，組織工学的に作製した軟骨の同種移植による軟骨再生に関する研究[11]，ならびに人工材料のスキャフォールドを使用しない軟骨細胞シート[5]および再生軟骨

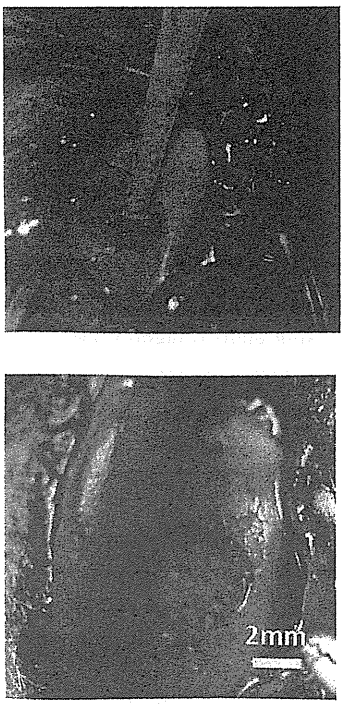
a



b



c



Primer（COL I）:
・forward: GCCTCGCTCACCACCTTCT
・reverse: CAATCTGGTTGTTCAGAGACTTCAG
Primer（COL II）:
・forward: GCAGCACGTGTGGTTTGG
・reverse: CAGGCTGCTGTCTCCATAGCT

d　（×40）

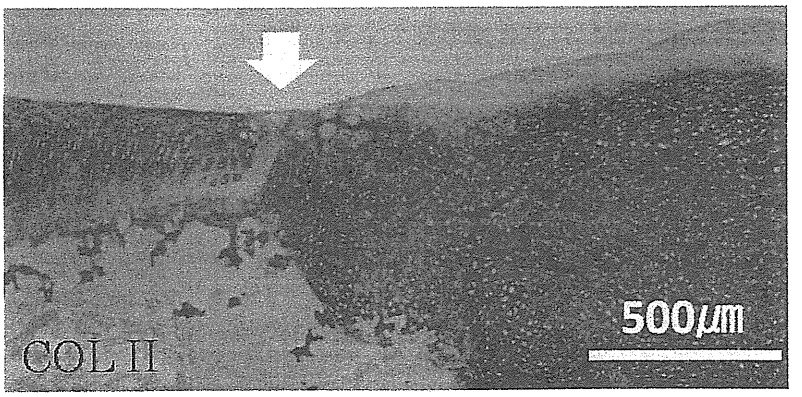


図2　結果

a：培養日数当たりSYとACの平均継代増殖率［(回収時細胞数/播種時細胞数)/(培養日数)］,
b：細胞スフェロイドの作製過程における遺伝子発現（RT-PCR）,
c：細胞スフェロイド移植の実際,
d：移植後4週における組織学的検討（Collagen type IIによる免疫組織染色）

plateによる軟骨修復・再生に関する研究[9]などの成果から，軟骨損傷を治療するためには，損傷部位へ動員されるMSCの存在と組織修復・再生に必要最小限の軟骨誘導イニシエーターの存在が必須条件であること，すなわち組織工学的軟骨の存在が重要であることを見出している．われわれの見解から推察すると，本研究の細胞スフェロイドでは，ACが軟骨誘導イニシエーターとしてSY由来のMSCと協働する．軟骨修復・再生に必要な最小の解剖的かつ生理学的な単位の細胞スフェロイドを治療単位として大量に移植することで広範囲の病変部においても最大の治療効果が期待できる．しかも，軟骨下骨まで到達しない部分欠損は，骨髄からのMSCの動員も少なく，その結果，組織修復・再生が十分ではなく，不完全な治療となる場合が多いが，今回の細胞スフェロイドは，上記の条件を満たすため，良好な軟骨修復・再生が期待できる．さらに，患者の正常軟骨部からの採取に制限のあるACの部分的な代替としてSYを用いることで移植体作製が容易となり，低侵襲での組織採取とその効率的な活用法として期待できる．

現在，ウサギの移植予備実験（図2c）において75%SY・25%ACで構成した条件の細胞スフェロイドの結果が良好な結果を得ている．この細胞スフェロイドの移植による移植後4週における欠損部位の組織学的な検討の結果（図2d）では，正常軟骨部位と移植部位の再生軟骨組織間の良好な接合と強く染色されたCollagen type 2の存在が確認され，移植部位は軟骨様形質を発現した組織で修復・再生されていた．

今後，さらに長期移植後の全層欠損モデルの結果や部分損傷モデルにおける治療効果を明らかにする予定である．

結　語

スフェロイド作製法により，ACIの新規移植体の作製が可能となった．ACとSYからなる本細胞スフェロイドは，高い増殖率を示すSYをACの部分的な代替として用いることで，短期間での大量作製が可能であった．新規移植体は今後ACIにおいて低侵襲での組織採取とその効率的な活用法として期待できる．

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