再生医療の基礎シリーズ―生医学と工学の接点―

編集委員会

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Modification of measurement methods for evaluation of tissue-engineered cartilage function and biochemical properties using nanosecond pulsed laser

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ABSTRACT

There is a demand in the field of regenerative medicine for measurement technology that enables determination of functions and components of engineered tissue. To meet this demand, we developed a method for extracellular matrix characterization using time-resolved autofluorescence spectroscopy, which enabled simultaneous measurements with mechanical properties using relaxation of laser-induced stress wave. In this study, in addition to time-resolved fluorescent spectroscopy, hyperspectral sensor, which enables to capture both spectral and spatial information, was used for evaluation of biochemical characterization of tissue-engineered cartilage. Hyperspectral imaging system provides spectral resolution of 1.2 nm and image rate of 100 images/sec. The imaging system consisted of the hyperspectral sensor, a scanner for x-y plane imaging, magnifying optics and Xenon lamp for transmmissive lighting. Cellular imaging using the hyperspectral image system has been achieved by improvement in spatial resolution up to 9 micrometer. The spectroscopic cellular imaging could be observed using cultured chondrocytes as sample. At early stage of culture, the hyperspectral imaging offered information about cellular function associated with endogeneous fluorescent biomolecules.

Keywords: regenerative medicine, tissue engineering, articular cartilage, fluorescence, hyperspectral, multispectral, tissue characterization, chondrocyte,

INTRODUCTION

1.1 Regenerative medicine of articular cartilage

There has been an increasing demand for development of technology that enables determination of the functions and components of engineered tissues in comparison to those of native tissues in order to establish reliable regenerative medicine, that is, the validation of regenerative medicine. The technology for validation should enable noninvasive evaluation of engineered tissues. It also requires that the validation of the repair process after transplantation and diagnosis of a disease before regenerative medicine is applied.

Regenerative medicine for articular cartilage, which is expected to be an effective therapy for osteochondral defect, osteoarthritis and rheumatism, is a very proactive clinical approach. Extracellular marix of articular cartilage is responsible for the major functional properties of cartilage. Consequently, characterization of extracellular matrix should be performed for evaluation of regenerative medicine for articular cartilage.

Articular cartilage is composed of scattered chondrocytes embedded in an abundant extracellular matrix. The matrix is mainly composed of proteoglycans and type II collagen, which consists of endogenous biomolecules. Fluorescence measurement can provide information about endogenous fluorescent biomolecules. We have developed a laser-induced auto-fluorescent spectroscopy system using the same excitation laser pulse as that used for viscoelastic measurement

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system for characterization of extracellular matrix, which has a possibility of simultaneous measurement together with the viscoelastic measurement system[1,2]. We proved that the auto-fluorescent spectrum enables discrimination of type II and type I collagen based on the principle that endogenous biomolecules have their own auto-fluorescent features[3].

1.2 Hyperspectral imaging

Hyperspectral imaging devices are common in remote sensing reconnaissance. It captures both spatial and spectral information in a single pixel[4]. Hyperspectral data sets are generally composed of over several hundreds continual spectral bands, whereas, multispectral data sets are usually composed of about 5 to 10 discrete bands of relatively large bandwidths (70-400 nm).

Hyperspectral imaging collects the same picture on many bands of the light spectrum to generate a "datacube" that can reveal objects and information that more limited scanners cannot pick up. Another advance of this form of imaging is that different elements leave unique spectral signatures behind in various bands of the spectrum. Using these specific signatures, it is possible to identify the materials that make up a scanned object. As the hyperspectral remote sensing combines imaging and spectroscopy in a single system, it often includes large data sets and require new processing methods.

The purpose of this study is to investigate the potential of the hyperspectral sensor technology for evaluation of effectiveness, validity and confirmation of regenerative medicine of articular cartilage.

MATERIALS AND METHOD

1.3 Hyperspectral imaging system

The hyperspectral imaging system is shown in Fig.1. The system mainly consisted of a hyperspectral sensor (AISA Eagle, Spectral Imaging Ltd.)[5], a scanner, magnifying optics, Xenon light (MAX-302, Asahi Spectra Co., Ltd) for transmmissive lighting and computer for control of image acquisition. The specification of hyperspectral sensor is summarized in Table 1. Relay lens, combination of lenses used to magnify the image obtained by the hyperspectral sensor. A scanner was set at 5.4 frames per second and the step-angle of 0.0375°.

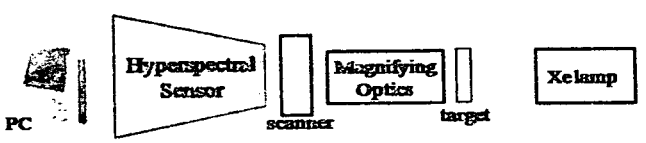

Fig.1 Hyperspectral imaging system

Table 1 Hyperspectral sensor specification

Spectral range	400-1000 nm
Spectral bands	512
Spatial pixels	1024
FOV	0.029° (0.51 mrad)

1.4 Sample target for hyperspectral image

(a) Transparent sheet for fundamental experiment

Transparent sheet printed with various color (blue, green, red et al.) by a printer, set resolution to 600 dpi (dots per inch) was used as sample, shown in Fig.2.

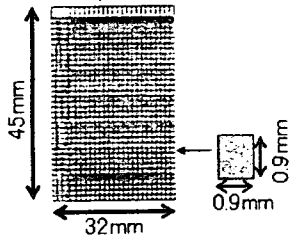

Fig.2 Imaging target for fundamental experiments using transparent sheet

(b) Cultured cells for actual cellular imaging

Articular cartilage separated from a knee joint obtained from a Japanese white rabbit weighing about 2.5 kg was digested for 4 hour in Dulbecco's modified Eagle's medium containing 0.0125% (w/v) collagenase P and 0.05% actinase E. The digested tissue was passed through a cell strainer with a pore size of 40 μm. The cells were then seeded and cultured in DMEM-F12 supplemented with 10 % fetal bovine serum. The part of cultured cartilage cells (chondrocytes) dyed with CSFE (absorption peak at 496 nm and fluorescence peak at 516 nm), which was purchased from DOJINDO and used without further purification. CSFE is deacetylated into a fluorescein derivative in a living cell and binds covalently to the amino group of the cellular components.

For the hyperspectral measurements, the cells were cultured in a chamber slide (LAB-TEK) at least one day in an incubator with air containing 5% CO_2 at 37°C. Just before the measurement, the unstained chondrocytes were washed with PBS and sealed without drying by a cover glass. As for the CSFE stained chondrocytes, 1mg/ml CSFE in dimethyl sulfoxide was added and left for 30-60 minutes. The chondrocytes were then washed and sealed as in the preparation of the unstained cells.

RESULTS AND DISCUSSION

1.5 Fundamental experiments

This experiment was performed to ensure the improvement of spatial resolution and retaining inherent spectral resolution. Precise alignment using the magnifying optics including a pair of relay lens achieved that it could be identified individual printer's dot at the printed transparent sheet for each color in the case of 600 dpi (dots per inch), showing Fig.3. The obtained image had 100 pixels per 0.9-mm-square at the sheet. Thus, the spatial resolution was finally calculated to be approximately 0.9 micrometer.

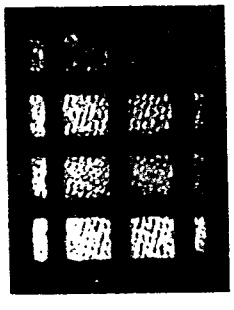

Fig.3 Obtained hyperspectral image of the imaging target. The image is converted into gray scale.

Spectrum per pixel could be obtained. The obtained spectra could be explained as follows. The illuminating light was absorbed at the printed sheet, and the hyperspectral sensor detected the absorption for each color. The performance of the obtained image is summarized in Table 2.

Table 2 Performance of the hyperspectral image

Spatial resolution	9 μm	Comparable microscopic image with x20 objective lens
Spectral resolution	1.2 nm	Inherent resolution
Homogeneous image area	3 mm x 3 mm	Improve by the scanner position

1.6 Cellular Experiments

Hyperspectral images could be obtained even though actual cultured cells were used as imaging targets. The CSFE-stained chondrocytes with monolayer culture are shown in Fig.4. Individual cell enable to be recognized without loss of the spatial resolution, as the size of chondrocytes are around 10-20 micrometer. The obtained image was comparable to fluorescence microscopic image with x20 objective lens. The obtained spectra reflected by the absorption and fluorescence of CSFE.

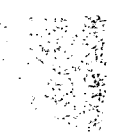

Fig.4 Obtained hyperspectral image of the CSFE-stained chondrocytes.

In the case of unstained chondrocytes with monolayer culture, the imaging enabled to be obtained as well as the CSFE-stained cells showing Fig.4. However, intrinsic fluorescent spectra, which contained autofluorescent features of endogeneous fluorescent biomolecule such as NADH and collagen as pericellular matrix, were unable to be observed. On the other hand, the intrinsic spectra could be recognized in the case of multilayer culture.

CONCLUSION

Hyperspectral sensor is revealed to be useful for evaluation of regenerative medicine of articular cartilage. This technique has a potential not only in vitro cultivation, but also in vivo application. In the case of in vivo application, monitoring after transplantation of engineered tissue would be valuable.

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Content

Multifunctional evaluation of tissue engineered cartilage using nano-pulsed light for validation of regenerative medicine

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Abstract— **There is a demand in the field of regenerative medicine for measurement technology that enables functions of engineered tissue to be determined. Articular cartilage, which has already been applied in clinical regenerative medicine, has specific mechanical functions. For meeting this demand, we previously proposed a noninvasive method for determination of the viscoelastic property based on photoacoustic measurements. However, characterization of the extracellular matrix of articular cartilage as well as determination of the viscoelastic property should be carried out for evaluation of regenerative medicine because the extracellular matrix plays an important role in the function of articular cartilage. The purpose of this study was to develop noninvasive measurement method for simultaneous evaluation of the viscoelastic properties by the photoacoustic method and characterization of the extracellular matrix of tissue engineered cartilage by the time-resolved autofluorescence spectroscopy. Third harmonic Q-switched Nd:YAG laser pulses were used as an excitation light source. Photoacoustic waves induced by the light pulses were detected using a piezoelectric transducer. The time-resolved fluorescence spectroscopy was obtained by using a photonic multichannel analyzer. The ratio of viscosity to elasticity measured by the photoacoustic method agreed well with the intrinsic viscoelastic parameters with a correlation coefficient of 0.98 when tissue-engineered cartilage tissues cultured for various periods (up to 12 weeks) were used as samples. There were significant differences in the measured fluorescent parameters among the culture conditions of cartilage because chondrocytes produce a specific extracellular matrix depending on its culture condition. The specific extracellular matrix contained a specific type of collagen such as collagen type I or type II, which each have specific fluorescent features. The combination of time-resolved autofluorescence spectroscopy and the photoacoustic measurement is expected to become a useful evaluation method in regenerative medicine of articular cartilage.**

Keywords— **Articular cartilage, Regenerative medicine, Tissue Engineering, Photoacoustic method, Time-resolved fluorescence spectroscopy**

I. Introduction

There has been an increasing demand in the field of regenerative medicine for development of non-invasive measurement technology that enables determination of the degree of similarity of engineered tissues to healthy native tissues in order to establish reliable methods for high-quality regenerative medicine.

Evaluation of engineered tissues of articular cartilage, which are used in clinical regenerative medicine for treatment of osteochondral defect, is currently carried out by histological, biochemical, and/or molecular biological analyses, which are invasive and do not enable consistent evaluation. Therefore, noninvasive technology for evaluation of engineered tissues of articular cartilage is required. Articular cartilage shows rheological behavior as viscoelastic material with time-dependent mechanical responses. Consequently, the viscoelastic properties should be determined for functional evaluation of articular cartilage. However, characterization of the extracellular matrix of articular cartilage as well as determination of the viscoelastic property should be carried out for evaluation of regenerative medicine because the extracellular matrix plays an important role in the function of articular cartilage.

The purpose of this study was to develop noninvasive measurement method for simultaneous evaluation of the viscoelastic properties by the photoacoustic method and characterization of the extracellular matrix of tissue engineered cartilage by the time-resolved autofluorescence spectroscopy.

II. Material and methods

A. Light source [1]

From a practical standpoint, a commercially-available 3rd (355-nm) harmonic Q-switched Nd:YAG laser (pulse width, 5-6 ns) was used as a nano-pulsed light source in this study. The light source was operated at a constant repetition rate of

10 Hz. The beam was focused with a lens and then coupled to a silica fiber with a core diameter of 400 μm. Transmitted light energy was maintained at approximately 30 μJ/pulse.

B. Photoacoustic method [2-5]

Stress waves induced by the light pulses were detected by a piezoelectric transducer consisting of polyvinylidene fluoride–trifluoroethylene [P(VdF/TrFE)] film, 4 mm in diameter and 55 μm in thickness. Output signals of the photoacoustic transducer were amplified with a low-noise amplifier (bandwidth, 1 kHz–100 MHz; gain, 46 dB) and acquired with a multichannel digital oscilloscope (bandwidth, 1 GHz). The relaxation time (τ), which was calculated as the time for the stress wave amplitude to decrease by a factor of 1/e, was measured. The relaxation time has a relationship with the viscous (η)-to-elastic (G) modulus rate (η /G=tan δ) when ω is defined as the frequency of a stress wave: $\tau = (\omega \cdot \tan\delta)^{-1}$.

C. Time-resolved fluorescent spectroscopy [6]

Time-resolved fluorescent spectroscopy was obtained by using a photonic multichannel analyzer with a 4-ch digital signal generator. The fluorescent features of the developed measurement system are as follows: wavelength range, 200-860 nm; wavelength resolution <3 nm; exposure time, 19 ms; gate time, 10 ns. The parameters of measured fluorescence, which were obtained using MatLab software, were peak wavelength at fluorescence maximum, fluorescent spectral bandwidth at half-maximal amplitude (FWHM), and integrated intensity of time-resolved spectrum.

D. *Cartilage collection and isolation of chondrocytes*

Knee joints were obtained from 4-week-old female Japanese white rabbits, each weighing about 1 kg. Articular cartilage was separated from the joint with a scalpel and digested for 4 h in Dulbecco' s modified Eagle's medium containing 0.0125% (w/v) bacterial collagenase P and 0.05% actinase E. The digested tissue was passed through a cell strainer with a pore size of 40 μm. The filtrate was centrifuged at 1500 rpm for 10 min to separate the cells.

E. Samples for photoacoustic measurement [7]

The cartilaginous cells were then seeded at high density (1 x 10^6 cells per scaffold) into an ACHMS scaffold (diameter, 11 mm; thickness, 2 mm) in 48-well plates by centrifugation at 500 rpm for 5 min and then cultured in DMEM–F12 supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2 in air and 100% relative humidity.

F. Samples for time-resolves spectroscopy

Articular cartilage separated from a joint that was obtained from a Japanese white rabbit weighing about 2.5 kg was used for an experiment on evaluation of normal cartilage. Intervertebral disc of the Japanese white rabbit was used as fibrous cartilage. Collagen types I and II offered commercially were used as components of the extracellular matrices of cartilages.

For monolayer culture, the cartilaginous cells were seeded in 48-well culture plates at cell densities of $2x10^5$/mL and incubated as described above.

III. Results and Discussion

Figure 1 shows plots of measured photoacoustic relaxation times as a function of cultured week. Smaller relaxation times were obtained for tissue-engineered cartilage cultured for a longer period, which was equivalent to greater elasticity and/or smaller viscosity. This experiment resulted in the reflection of synthesis process of the extracellular matrix such as collagen and proteoglycan which directly relate cartilage viscoelasticity, with increased cultured week.

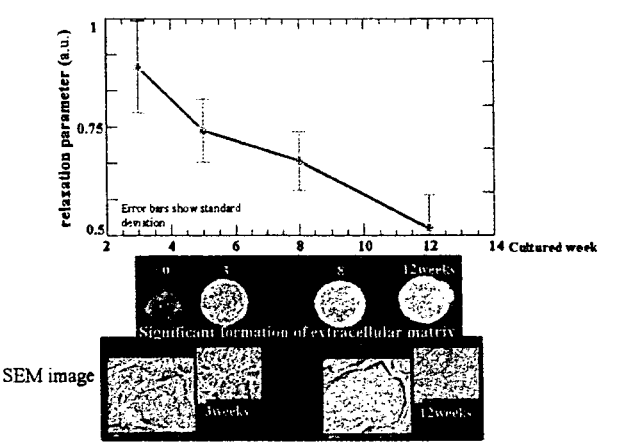

Fig.1 Comparison between relaxation times and photos, SEM (scanning electron micrograph) of tissue-engineered cartilages. [4]

Autofluorescent spectral bandwidth of articular cartilage (65 nm) is narrower than that of intervertebral disc (120 nm). The fluorescent spectrum of collagen type II differed from that of collagen type I. The obtained parameters of auto fluorescent spectrum of articular cartilage were similar to those of collagen type II. And, those of collagen type I were similar to those of intervertebral disc. Since structure material of articular cartilage is collagen type II and that of intervertebral disc is mostly collagen type I, the difference between fluorescent spectra of articular cartilage and intervertebral disc was caused by the difference in collagen type II and collagen type I. Therefore, the compositional difference of articular cartilage and intervertebral disc enables characterization by autofluorescent spectroscopy.

The autofluorescent spectrum of the monolayer culture showed variation with various culture days. The tissue-engineered cartilage of the monolayer culture was gradually dedifferentiated and changed its phenotype during cultivation. The extracellular matrix such as collagen was produced depending on its phenotype. The spectrum of the monolayer culture was reflected of the phenotypic change. Therefore, tissue-engineered cartilage can be characterized using autofluorescent spectroscopy.

IV. Conclusions

The combination of time-resolved autofluorescence spectroscopy and the photoacoustic measurement is expected to become a useful evaluation method in regenerative medicine of articular cartilage.

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