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Development of a Diagnostic System for Osteoarthritis Using a Photoacoustic Measurement Method

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Background and Objectives: We demonstrated that photoacoustic measurement enables viscoelastic characterization of biological tissue. The purpose of this study was to develop a practical photoacoustic measurement system for diagnosis of osteoarthritis (OA) by viscoelastic characterization of articular cartilage.

Study Design/Materials and Methods: The portable system consists of a commercially available 3rd harmonic Q-switched Nd:YAG laser as a light source and a transducer, which is arranged coaxially with an optical fiber. Cell proliferation tests were performed to study the effect of laser irradiation on chondrocytes. Photoacoustic measurements were performed using enzymatically treated cartilage as a model of OA.

Results: There was no significant damage of chondrocytes caused by laser irradiation (100 μJ/mm^2, 5 Hz, 30 shots). The change in relaxation times measured by the photoacoustic measurement had a positive correlation with time of enzymatic treatment, that is, the degree of cartilage degeneration.

Conclusions: We have developed a noninvasive photoacoustic measurement system designed for arthroscopic use and have demonstrated the applicability of this system to the diagnosis of OA-like cartilage degeneration. Lasers Surg. Med. 38:249–255, 2006. © 2006 Wiley-Liss, Inc.

Key words: 3rd harmonic Q-switched Nd:YAG laser; cartilage degeneration; cell proliferation test; relaxation time; viscoelastic characterization

INTRODUCTION

Osteoarthritis (OA) is a common musculoskeletal disorder affecting millions of individuals worldwide. OA, a progressive disease of articular cartilage, is associated with severe joint pain and results in joint immobilization [1]. Currently, there is no complete cure for OA, but early detection followed by efficient therapy can slow its detrimental effects. A diagnostic tool that enables early and reliable diagnosis of this degenerative joint disease is needed [2]. One of the first signs of cartilage degeneration is tissue softening, which can occur before any histological changes are observed. With time, softening can lead to cartilage fibrillation and OA. Before any disruption of the collagen meshwork occurs, the changes are believed to be reversible [3,4]. For preventive actions, it would therefore be desirable to detect tissue changes in the earliest stages of OA [5]. There have been numerous studies on the usefulness of the noninvasive technique of magnetic resonance imaging (MRI) to study cartilage structure and degeneration both in vitro and in vivo [6,7]. However, the MRI imaging technique cannot accurately evaluate quantitative property of articular cartilage. In arthroscopy, the cartilage condition is typically evaluated visually and by manually palpating the hardness of the articular surface with a blunt probe. At the present time, there are only few quantitative in vivo devices available that enable accurate evaluation of the condition of cartilage, which shows rheological behavior as viscoelastic material [8,9]. Quantitative measurement methods of viscoelasticity, which is directly related to the major function of articular cartilage, are needed for thorough and accurate diagnosis and for follow-up study of cartilage degeneration and repair.

We found that tissue viscoelasticity affected propagation and attenuation of the stress wave induced by pulsed laser irradiation [10]. Its relaxation time, calculated as the time at which the amplitude of the stress wave decreased by a factor of $1/e$, gave the intrinsic relaxation parameters (η/G) of the tissue, where η is viscosity and G is elasticity. We accordingly proposed a method for viscoelastic characterization of biological tissue using photoacoustic measurements. We have already reported that relaxation times measured by the photoacoustic method were in agreement

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with intrinsic viscoelastic properties for gelatin models as tissue phantoms [10,11]. Relaxation times measured by the photoacoustic method were in agreement with intrinsic viscoelastic parameters with a correlation coefficient of 0.98 when engineered cartilage tissues cultured for various periods (up to 12 weeks) were used as samples [12,13]. The advantages of photoacoustic measurements include the capability of noninvasive, practical, real-time, and in situ measurements. Another advantage is controllable depth sampling, by which specific mechanical characterization of articular cartilage of a few millimeters in thickness covering the ends of articulating bones can be performed without the influence of the subchondral bone, which has a very different viscoelasticity.

The first purpose of this study was to develop a noninvasive and practical system for diagnosis of OA using photoacoustic measurement that enables characterization of viscoelasticity. Photoacoustic measurements were performed using enzymatically digested cartilage as a model of OA in vitro.

MATERIALS AND METHODS

Photoacoustic Measurement System

The experimental set-up is shown in Figure 1A. A nanosecond pulse light was used for the excitation of stress waves. The light beam was focused with a lens and then coupled to a silica fiber of 600 μm in core diameter. Stress waves induced by the light pulses were detected by a piezoelectric transducer consisting of a poly(vinylidene fluoride-trifluoroethylene) (P(VdF/TrFE)) film of 55 μm in thickness (Fig. 2). Output signals of the photoacoustic transducer were amplified with a low-noise field-effect transistor (FET) amplifier (bandwidth, 300 Hz–100 MHz; gain, 46 dB, SA-220F5, NF Electronic Instruments, Yokohama, Japan) and acquired with a multi-channel digital oscilloscope (bandwidth, 1 GHz, TDS 3054B, Textronics, Beaverton, OR).

The relaxation time theoretically has a relationship with viscous-to-elastic modulus rate [14]. The relaxation time (τ) was calculated by using the Levenverg–Marquardt algorithm, a nonlinear least-squares method, as follows. When attenuation of stress wave intensity is affected only by reflection at boundaries and relaxation during transmission through viscoelastic materials, the time course of the stress wave intensity is expressed by the following equation.

$$I_\delta = I_0 \times R \times \exp(-\delta t/\tau),$$

where τ is relaxation time, I_0 is initial intensity of the stress wave, R is the product of the reflectance at the front surface and posterior boundary, δt is time required to transmit the stress wave back and forth between the sample and I_δ is the first peak intensity of each wave packet of measured acoustic pulse sequence at $t = \delta t$. We investigated the characteristics of the measured signal to determine whether the diffraction affected the calculation of relaxation time from the measured stress wave intensity. It was shown that there was no necessity to consider the diffraction effect in the case of measurement of articular cartilage of a few millimeters in thickness in comparison to the diffraction length (>20 mm).

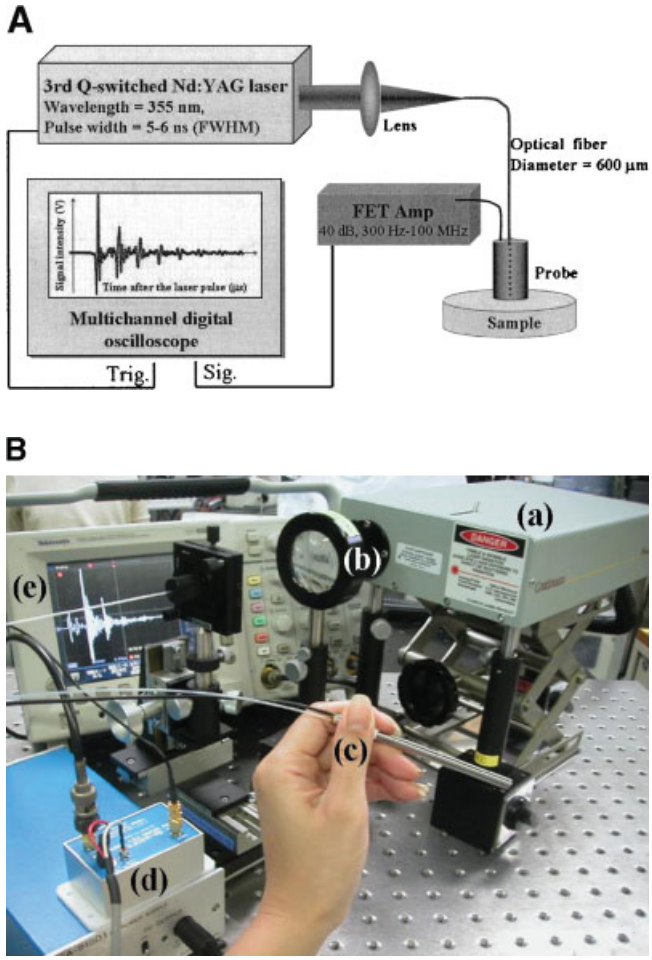

Fig. 1. **A**: The system for photoacoustic measurement. The lens is a silica lens with a 70-mm focal length, and the field-effect transistor (FET) amplifier is used to amplify the photoacoustic signal. The monitor of the digital oscilloscope shows a typical observed waveform of the photoacoustic signal. Trig., photoacoustic signal trigger; Sig., photoacoustic signal. **B**: Photo of the assembled system. (**a**) laser, (**b**) lens, (**c**) probe, (**d**) FET amplifier, (**e**) digital oscilloscope.

Assembly of Diagnostic System

Figure 1B shows the entire portable photoacoustic measurement system. For determination of the light source from a practical standpoint, a commercially available 3rd (355 nm) harmonic Q-switched Nd:YAG laser (pulse width (FWHM), 5–6 ns; Minilite II, Contimuum, Santa Clara, CA) was used in this study. The laser was operated at a repetition rate of 5 Hz, which coincided with the previous study. In the previous study, a 250-nm laser pulse, which was tuned by an optical parametric oscillator (pulse width

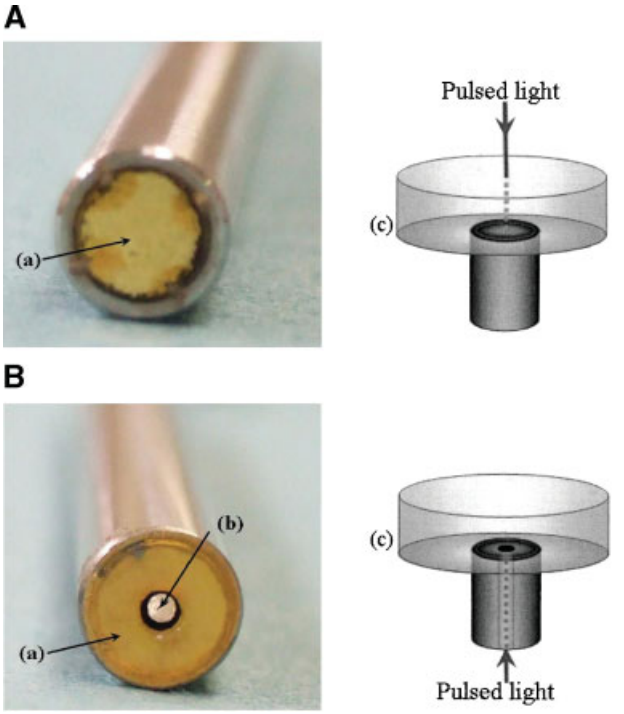

Fig. 2. **A**: The opposite probe and (**B**) the coaxial probe. (**a**): sensor element, (**b**) optical fiber, (**c**) sample. The coaxial probe arranges the optical fiber coaxially with the ring-shaped sensor transducer. The black element is the sensor element consisting of a poly(vinylidene fluoride-trifluoroethylene) (P(VdF/TrFE)) film. The coaxial probe can be sterilized for clinical use.

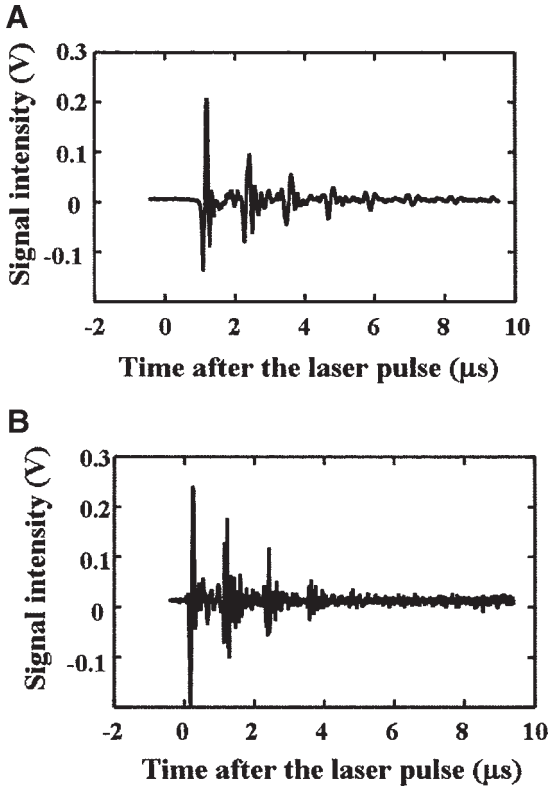

Fig. 3. Transient recorded photoacoustic signals from gelatin of 20% in density. The measured waveform in (**A**) was detected by the opposite probe. The waveform in (**B**) was detected by the coaxial probe.

(FWHM), 5–7 ns; Quantra-Ray MOPO-710 and Quantra-Ray GCR-290; Spectra Physics, Mountain View, CA) was used as the light source [10]. The relaxation times measured using a 355-nm laser pulse were compared with those using a 250-nm laser pulse in our previous study in order to confirm the applicability of 355 nm as an excitation light source. Gelatin phantoms of various densities and porcine articular cartilage were used as samples. The gelatin samples were prepared in the following way: a solution of gelatin in water was adjusted to specified densities with a lipid-emulsifying agent (Intralipos 20%, Otsuka Pharmaceutical, Kanda, Japan) as a scatterer. The density of gelatin was varied in the range of 5–25%, which covers the density range of collagen in biological tissue. The thicknesses of the gelatin samples were fixed at approximately 1 mm.

The transducer was designed for used in arthroscopy. In our previous study, the optical fiber was placed opposite to the transducer (opposite probe) [10–13]. The designed transducer for arthroscopic application was set coaxially with an optical fiber (coaxial probe). Schematic drawings of the opposite probe and coaxial probe are shown in Figure 3A, B. The relative locations of the optical fiber and the sensor transducer were optimized when the output end of the fiber was arranged at the center of the transducer (i.e., the optical fiber was coaxially arranged with the ring-shaped sensor transducer for in situ diagnosis). The probe was 6 mm in outer diameter and 5 mm in inner diameter, and the outer casing was 15 cm long. Photoacoustic measurements were performed using the coaxial probe as they were with the opposite probe to determine whether or not the coaxial probe could detect photoacoustic signals.

Cell Proliferation Test

The effect of laser irradiation on cartilaginous tissue was studied by using a cell proliferation test. Knee joints were obtained from 4-week-old female Japanese White rabbits each weighing approximately 1 kg. Articular cartilage was separated from the joint with a scalpel and digested for 4 hours in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 0.0125% (w/v) bacterial collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.05% actinase E (Kaken Pharmaceutical, Tokyo, Japan). The digested tissue was passed through a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) with a pore size of 40 μm. The filtrate was centrifuged at 1,500 rpm for 10 minutes to separate the cells. The cells were then seeded at a high density (1×10^6 cells/scaffold) in 96-well plates (Sumitomo Bakelite, Tokyo, Japan) by centrifugation at 500 rpm for 5 minutes and then cultured in DMEM/F12 (Iwaki, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37°C

in an atmosphere of 5% CO_2 in air and 100% relative humidity. These cell culture procedures were performed in a way similar to that used in a previous study [12]. Cells subjected to irradiation with 355-nm laser pulses were divided into five groups: those subjected to irradiation of 100 $\mu J/mm^2$, 30 shots, which is a clinically used condition (group A); those subjected to irradiation of 150 $\mu J/mm^2$, 30 shots (group B), which is the maximum laser energy of this system; those subjected to irradiation of 100 $\mu J/mm^2$, 1,500 shots (group C); those subjected to immersion in alcohol (group D) as a negative control; and a control without irradiation (group E). The laser pulse energy was adjusted with a variable attenuator and measured with a power meter (PE10; Ophir Japan, Saitama, Japan). The laser beam diameter was adjusted to the diameter of the cell-culture plate. Cell viability was evaluated by using a cell counting kit-8 after laser irradiation to the cultured cells.

Cell proliferation was determined by a WST-8 assay using the cell counting kit-8 (CCK-8; Dojindo Molecular Technology, Gaithersburg, MD) [15]. The assay was carried out according to the manufacturer's instructions [16–18]. A 96-well plate containing cells from each of the five groups was inoculated with 10 μl of prepackaged CCK-8 solution. They were incubated at 37°C in an atmosphere of 5% CO_2 in air and 100% relative humidity up to 1 hour. Absorbance at 450 nm of each well was measured using a microplate reader (MTX Lab System Inc., Vienna, VA).

Model of Degeneration of Articular Cartilage

OA is caused by loss of proteoglycans, degradation of the collagen network, and an increase in fluid content [19]. Experimental treatments using specific enzymes such as trypsin can simulate these changes [20]. In the present study, trypsin treatment was used for cartilage degeneration as an OA model. Cylindrical cartilage-bone plugs (12 mm in diameter, $n = 20$) were prepared for this experiment from the patellae of pigs. Porcine knee joints were delivered intact from a local slaughterhouse within 1 hour after slaughter. Sample plugs were initially incubated under physiological conditions (37°C, 5% CO_2) in saline. The enzyme addition was used for experimental degradation of the tissue matrix using trypsin in phosphate-buffered saline (1 mg/ml) to degrade primarily proteoglycans [19,21]. The treatment time was varied up to 24 hours to control the extent of degeneration. Since trypsin treatment induces biomechanical changes, we performed photoacoustic measurements for biomechanical evaluations. The samples were fixed in 10% formalin solution after the measurements for histological study. The samples were sectioned at 4 μm in thickness for microscopic observation with toluidine blue staining.

RESULTS

Assembly of the Diagnostic System

The waveforms measured by using the opposite probe and the coaxial probe are compared in Figure 3A, B. The photoacoustic signals were measured in the form of acoustic pulse sequences that is due to relaxation of the laser-induced stress wave during propagation through the sample and multiple reflections at acoustic boundaries of the sample. In the waveforms shown in Figure 3A, B, there are pulse sequences with the same tendency except for the first wave packet as shown in Figure 3B measured by the coaxial probe. This first wave packet of the waveform is caused by a surface wave. The surface wave propagation was detected directly by the coaxial probe without a pathway through the sample. The subsequent wave packets of the waveform measured using the coaxial probe corresponded to the wave packets sequence measured using the opposite probe, proving that the signal measured by the coaxial probe corresponded to the photoacoustic signal induced by the laser irradiation. In Figure 3A, there are five wave packets and the peak intensity of each wave packet was used for the calculation of relaxation time. In Figure 3B, there are four wave packets and the peak intensity of each wave packet was used for the calculation of relaxation time. Relaxation times were acquired from photoacoustic signals with single pulse irradiation. The relaxation times measured by excitation wavelengths of 355 and 250 nm are compared in Figure 4. In the case of gelatin samples, it is shown that a gelatin model of greater density resulted in shorter relaxation times. In the case of the porcine articular knee cartilage, the relaxation times were in the range of those in the gelatin densities between 12.5% and 30%. The relaxation times obtained by the excitation wavelength of 355 nm correlated with those obtained by the excitation wavelength of 250 nm with a correlation coefficient of about 0.90.

Cell Proliferation Test

Cells subjected to irradiation with 355-nm laser pulses were divided into five groups: those subjected to irradiation of 100 $\mu J/mm^2$, 30 shots (group A); 150 $\mu J/mm^2$, 30 shots (group B); 100 $\mu J/mm^2$, 1,500 shots (group C); immersion in alcohol (group D) as a negative control; and a control without irradiation (group E). The experimental results are shown in Figure 5. The results for group D are statistically different from those for all other groups. The results for group E showed no significant differences from those for the other laser irradiation groups (groups A–C).

Photoacoustic Measurements of a Degeneration Model of Articular Cartilage

Figure 6 shows relaxation time as a function of trypsin treatment time. There was a positive correlation between relaxation time and trypsin treatment time. Longer treatment time resulted in longer relaxation time, which is equivalent to greater viscosity and/or smaller elasticity. There were significant statistical differences between results for cartilage specimens treated with trypsin for 0 hour and 6 hours, those treated for 6 hours and 12 hours, and those treated for 12 hours and 24 hours.

Histologically, the normal cartilage specimen (Fig. 7A) showed a layered toluidine blue staining pattern from the surface to the boundary of bone. In contrast, the trypsin-treated cartilage demonstrated that longer treatment time

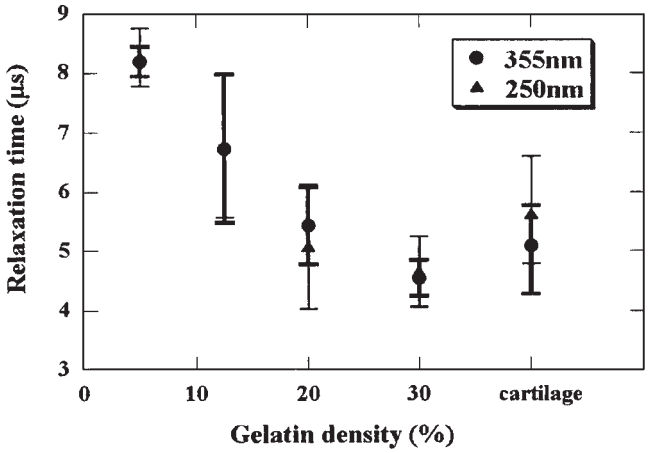

Fig. 4. Comparison of relaxation times measured by the photoacoustic measurement in the case of 355- and 250-nm excitation as a function of gelatin density. The plots at the far right correspond to the relaxation times of the cartilage. Closed circles represent relaxation times in the case of 355-nm excitation, and triangles represent relaxation times in the case of 250-nm excitation. Error bars show standard deviation (n = 4).

showed a larger loss of staining. The histological image of cartilage specimens treated with trypsin for 12 hours showed complete loss of staining (Fig. 7B).

DISCUSSION

Assembly of the Diagnostic System

Laser-induced stress waves, which consist of a compression wave and a rarefaction wave, can be induced under the following condition: $\tau_p \ll \frac{1}{\mu a \times V_s}$ where Vs is sound velocity of the sample, τ_p is pulse duration of the light, and μ_a is the absorption coefficient. The measured signals shown in Figure 3A, B enabled confirmation that they corresponded to the behavior of the laser-induced bipolar stress waves during propagation through the sample as well as the multiple reflections at the acoustic boundaries. In Figure 3B, the time at which the second peak appears coincides with the propagation time of the stress wave through gelatin of approximately 1 mm in thickness and reflections at the back and front surfaces when measured sound velocity of 1,550 m/s was used [22]. Therefore, photoacoustic measurement could be performed using the coaxial probe. Moreover, as the coaxial probe was only 6 mm in diameter, it is easy to use for arthroscopy.

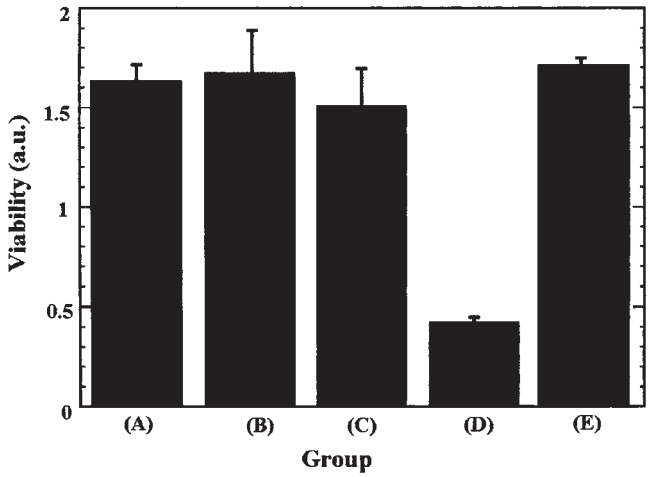

Fig. 5. Results of a cell proliferation assay using WST-8. The data were obtained 1 hour after incubation and inoculation. Group A: 100 μJ/mm^2, 30 shots, group B: 150 μJ/mm^2, 30 shots, group C: 100 μJ/mm^2, 1,500 shots, group D: negative control (immersion in alcohol), group E: control without irradiation. Error bars show standard deviation ($n = 4$–6).

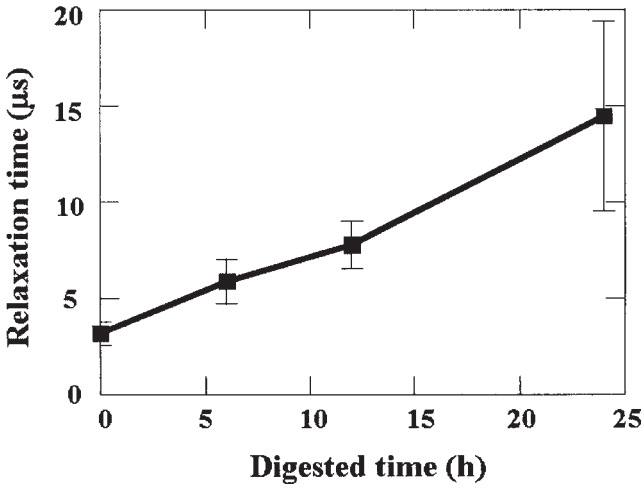

Fig. 6. Relaxation times measured by the photoacoustic measurement method as a function of trypsin treatment time (hours). Error bars show standard deviation ($n = 4$–6).

Figure 4 shows that the gelatin model of greater density gives shorter relaxation times in the case of 355-nm excitation. This tendency was also similar to that of the 250-nm excitation. The gelatin model of great density corresponds to collagen of great density. The relaxation time of the articular cartilage was in the range of 12.5–30% of gelatin density. In the case of articular cartilage, the extracellular matrix, which mainly consists of collagen and proteoglycans, is directly related to the function (viscoelasticity) [23]. Since the change in relaxation times of gelatin with various densities corresponded to the change in collagen density, these results demonstrated the possibility for detection of change in function, that is, diagnosis of degeneration of articular cartilage.

Cell Proliferation Test

Although there were significant statistical differences between the results for group D and those for the other groups, there were no significant differences between results for groups A–C and E, indicating that there was no noticeable damage to chondrocytes caused by the laser irradiation (Fig. 5). In this cell proliferation assay, naked cultured cells were used as samples, since destruction of the

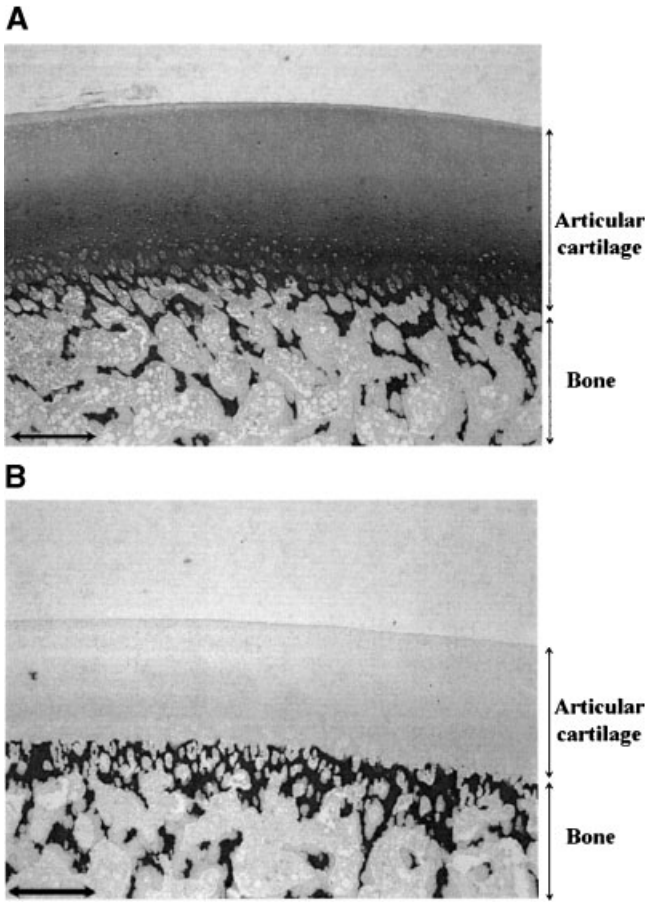

Fig. 7. Results of the histological study. Scale bar = 0.75 mm. **A**: Toluidine blue staining of a normal porcine cartilage specimen. **B**: Toluidine blue staining of a cartilage specimen treated with trypsin for 12 hours. The specimen shows an extensive loss of proteoglycans in the tissue.

extracellular matrix has already occurred in the case of advanced OA. The naked cells were more sensitive to external stress than tissue with abundant extracellular matrix such as cartilage, which normally has only 2–3% cell density with abundant extracellular matrix. In the present study, even using naked cells, there were no significant effects of laser irradiation on cell proliferation using the current irradiation application. Therefore, the photoacoustic measurement method with the current irradiation application was confirmed to be a noninvasive and thus a safe method.

Photoacoustic Measurements of Models of Degeneration of Articular Cartilage

Lyyra et al. reported that enzyme digestion produced changes in the structure and constitution of the extracellular matrix [5]. Trypsin treatment of the tissue caused remarkable loss of proteoglycans in the cartilage as shown in Figure 7. Changes in the extracellular matrix simultaneously caused change in viscoelasticity of the cartilaginous tissue. The degree of change in viscoelasticity corresponded to the degree of change in the extracellular matrix (i.e., the degree of degeneration). They also reported that treatment with trypsin (50 μg/ml) for 24 hours resulted in a decrease in the stiffness of digested cartilage by 36% [5]. Suh et al. reported that treatment with trypsin (1 mg/ml) for a period of several hours resulted in a decrease in digested cartilage stiffness by 46% [19]. In the present study, the difference in the relaxation times with various trypsin treatment times seems to reflect the various degrees of degenerations of cartilaginous tissue. A comparison of the measured mechanical properties with data obtained by Lyyra et al. and Suh et al. was difficult because measured parameters were different and the diffusion of trypsin depends not only on trypsin density and its treatment time but also on the size of the sample. However, there was an agreement in which higher density and longer treatment time resulted in a greater change in mechanical properties. The results therefore indicated that the photoacoustic measurement enables diagnosis of cartilage degeneration. From the viewpoint of early diagnosis, the measurement method that we have developed has a distinct advantage because a statistically significant difference was found between normal cartilage and cartilage digested for 6 hours. These results demonstrate the usefulness of using photoacoustic measurements for viscoelastic characterization.

From a diagnostic point of view, it is essential to determine cartilage viscoelasticity in vivo because different cartilage pathologies affect the structure and, consequently, the mechanical properties of the tissue. In OA, there is a loss of proteoglycans and fibrillation of the collagen network, which induce changes in viscoelastic properties of the tissue. The measured parameter is relaxation time, which corresponds to the viscosity-elasticity ratio using photoacoustic measurement. The current measurement system can only show the ratio and not the absolute values of viscosity and elasticity. If it is necessary to measure the absolute viscoelastic value for OA diagnosis, a load cell with a micrometer will just have to be connected to the current coaxial probe. A load cell, which is a kind of loading sensor, is used for indentation tests. An indentation system for the assessment of biomechanical properties, especially elastic properties of tissues, has been developed [24]. However, data for relaxation times obtained in a previous study [12] and in the present study have revealed that relaxation time is a good indicator of the status of the extracellular matrix of cartilage. In the case of the tissue engineering process, the relaxation time is a good indicator of extracellular matrix formation [12], and in the degenerative process, the relaxation time was shown in the present study to be a good indicator of the degenerative process. Therefore, the relaxation time may be the best indicator of the status of the cartilage viscoelastic function. Moreover, arthroscopic measurement of cartilage viscoelasticity may be more useful in providing information about the degenerative conditions of cartilaginous tissue than X-ray images of knee joint space from the viewpoint of OA diagnosis. We have developed a portable noninvasive photoacoustic measurement system for OA diagnosis. Using a degenerative model

of a porcine cartilage-bone plug, the photoacoustic measurement revealed the possibility of OA diagnosis. We conclude that our measurement method, designed for arthroscopic use, can detect viscoelastic property changes related to cartilage degeneration.

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