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Measurement System for Biomechanical Properties of Cell Sheet *

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Abstract— **In this study, we present a new fixture (self-attachable fixture) and tensile test system for measuring mechanical properties of cell sheet. To evaluate strength of cell sheet, it is the most important to measure mechanical properties of tensile mode. However, there has been no study which measured the tensile mechanical properties of cell sheet, since it has been difficult to attach a cell sheet in the tensile test system owing to the structure of the conventional fixture, and there has been no tensile test system which had a measurement range that covered the tension force range of the cell sheets. Therefore, we have addressed these problems by developing a self-attachable fixture and a tensile test system. By using developed system, we measured mechanical properties (tension, stress and initial stiffness) C2C12 of cell sheet cultured in different recipe of culture medium. The initial stiffness of cell sheet cultured in culture medium without FBS had a tendency to become stiffer. This indicates that our new fixture and test system are applicable for evaluating mechanical properties of cell sheets.**

I. Introduction

In the regenerative medicine, cell sheet engineering [1-4] is one of the most promising approaches. To use cell sheet in regenerative medicine, it is important to evaluate quality of cell sheet. When evaluating quality of cell sheet, there have been some approaches such as morphological examination or immunofluorescence analyses [5]. In this study, we focus mechanical properties of cell sheet for evaluating quality of cell sheet. It is possibl that to apply evaluation by mechanical properties of cells, we can get much knowledge which is not gotten by morphological examination or immunofluorescence analyses. For example, we have to evaluate quality of cell sheet from the view point of strength of materials. Since cell sheet is extremely fragile, it is concerned that breakage of cell sheet in medical handling, and we are not able to use broken cell sheet for regenerative medicine. By evaluating mechanical properties of cell sheet, we can get optimal culture conditions for satisfied strength of cell sheet.

Uchida et al. measured the stiffness of the cell sheet in a state close to non-invasively using a hand air jet [6]. This method can measure mechanical properties of thickness mode, but it is not able to measure mechanical properties of tensile mode (spread direction of cell sheet). To evaluate strength of cell sheet, it is the most important to measure mechanical properties of tensile mode. In this study, we applied tensile test [7] for evaluating mechanical properties of cell sheet. The tensile test can measure mechanical properties of tensile mode of cell sheet.

When applying the tensile test to cell sheets, there were two problems owing to the structure of the fixtures that hold the sheets. First, it is difficult to attach the cell sheet in the test system by conventional fixtures due to the mechanical fragility of the sheet. In the tensile test of tissues or biomaterials, specimens are attached to the test system by clamping [8, 9, 10], or using pins or staples [11, 12, 13]. These methods cannot be used for cell sheets; for the clamp type fixture, the cell sheet may be squashed by the stress of clamping and for pin or staple type fixtures, the cell sheet will tear at the through holes used for the hooking pins or staples. Second, because the cell sheet contracts immediately when it is detached from the culture surface due to cytoskeletal tension and reorganization [14], it is difficult to load the same strain rate for every cell sheet in the tensile test.

To solve these problems, we developed a self-attachable fixture. The self-attachable fixture has many hooks aligned in

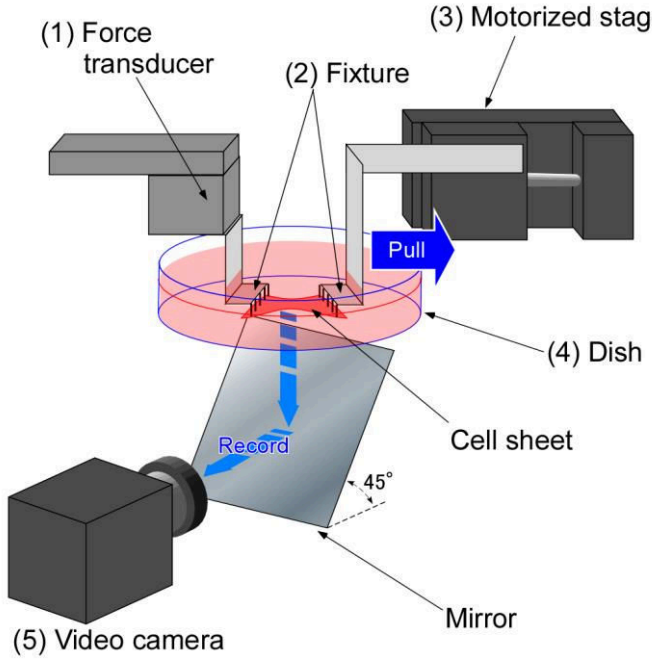

Figure 1. Schematic illustration of the developed tensile test system for measuring mechanical properties of cell sheets. The system consisted of five parts. (1) A force transducer to measure mechanical properties of the cell sheet. (2) A fixture which attaches the cell sheet to the test system. (3) A motorized stage that loads tensile force to the cell sheet. (4) A culture dish in which the cell sheet was cultured; it also protects the cell sheet from drying out during the tensile test. (5) A video camera for recording conditions during the test.

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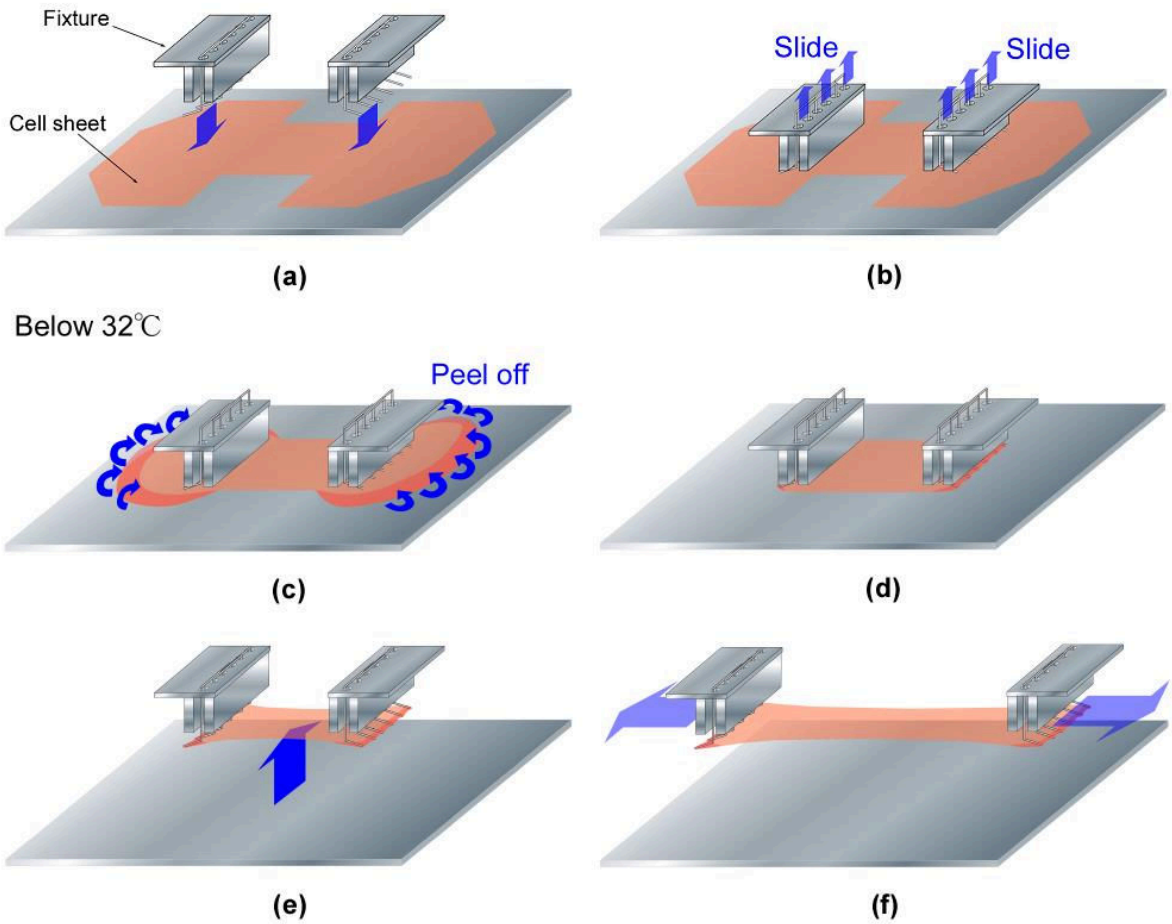

Figure 2. Schematic diagram showing the process to attach the cell sheet using the self-attachable fixture. (a) Cells were cultured at 37.C on a polystyrene dish coated with thermo responsive polymer. After forming the cell sheet, the self-attachable fixture was set on the cell sheet (b) and the semi-fixed hooks could slide in the vertical direction easily. Below 32 °C, (c) the culture surface became hydrophilic and the cell sheet lifted up. Then, (d) the cell sheet contracted and curled around the hooks due to cytoskeletal tension and reorganization, and finally it attached to the self-attachable fixture. After confirming cell sheet attachment, (e) the whole cell sheet was lifted from the culture surface and (f) the tensile test was performed.

a straight line for suppression of the stress concentration, and a force transducer can be hooked to the cell sheet without piercing the sheet. Furthermore, the self-attachable fixture attaches the cell sheet without its contraction. Because there is no tensile test system whose measurement range covers the tension range of cell sheets, we also developed a new tensile test system. By using developed system, we confirmed change of mechanical properties when changing FBS content of culture medium.

II. Materials and Methods

A. Test System

To measure mechanical properties of cell sheets, we applied the tensile test which has been widely used for measurement of mechanical properties [7]. Our tensile test system consisted of the five parts indicated in Fig. 1. (1) A force transducer (TMFMD-01, Tech Alpha, Japan) to measure mechanical properties of the cell sheet. (2) An actuator that loads tensile force onto the cell sheet; the cell sheet is stretched in the tensile direction at a constant speed using a computer-controlled electrical actuator (motorized stage: TSDM60-20X, SIGMA, Japan) (motor driver: GDB-5F30V1, Melec, Japan) (motor controller: NI7330 LabVIEW, National Instruments, United States). (3) A fixture which attaches the cell sheet to the force transducer and actuator. (4) A culture dish in which the cell sheet is cultured and which protects the cell sheet from drying out during the tensile test. (5) A video camera for recording the conditions of the test.

Measured tension was obtained by the force transducer as a transduced voltage signal and stored using a data acquisition system (PCI-6036A LabVIEW, National Instruments, United States). Stored data were analyzed with Igor Pro (Wave Metrics, United States). To synchronize the measurement data and video data, an LED lamp was set in the area of filming, and the voltage signal of the LED was input to the data logger. To avoid the blind area caused by the fixture when filming, we used a mirror which allowed us to record from the bottom of dish.

B. Design of Self-attachable Fixture

Generally, in a tensile test, a sample is attached to the force transducer by a clamping [8, 9, 10], pin or staple loading [10, 11, 12]. However, it is difficult to attach cell sheet by conventional fixtures from two reasons. First is fragility of cell

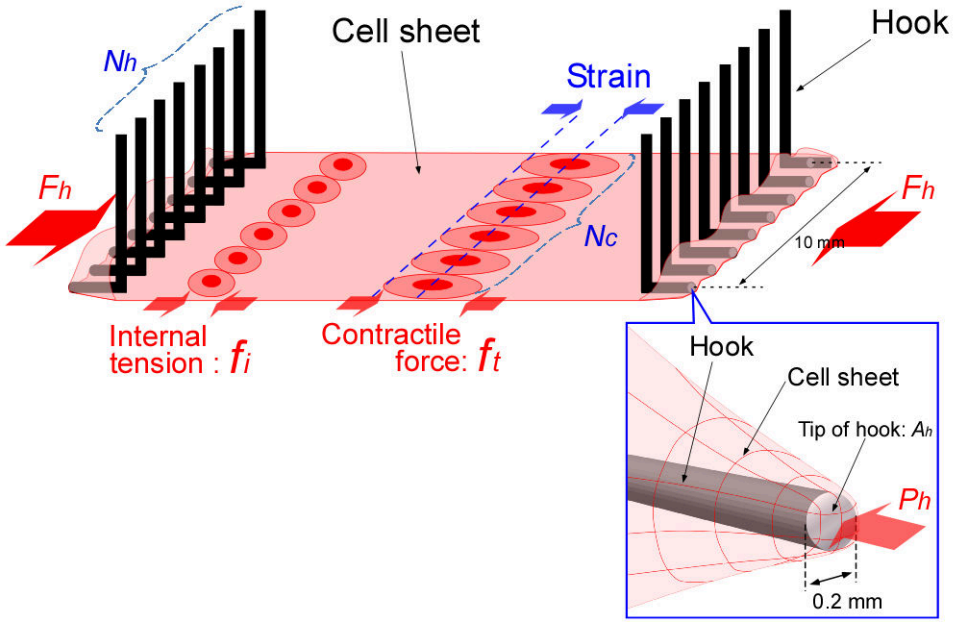

Figure 3. Schematic diagram of distribution of force at the self-attachable fixture.

sheet. For example, to clamp cell sheet without slipping, the cell sheet will be squashed because of clamping force is too strong. To use pin or staple loading has also problem that tear starts from through hole. Additionally, characteristics that cell sheet detached from substrate tend to folded or crumpled immediately. It is complex to unfold folding or crumpling of cell sheet. Second, when perform tensile test, it is difficult to keep size of cell sheet because of cell sheet is contracted when cell sheet is detached from culture surface due to cytoskeletal tension and reorganization [13]. Thus, it is difficult to apply same strain rate at every cell sheet. To apply conventional fixture for attaching, it is needed to detach cell sheet from culture surface. Therefore, we newly developed fixture which could attach cell sheet without breaking and contracting of cell sheet.

In our previous study, we have overcome first problem by developing multi hook type fixture [14]. The multi hook type fixture has several hooks and can attach cell sheet by hooking without piercing it. This method need to hook cell sheet by the hand, and could not solve second problem. We improved multi hook type fixture; self-attachable fixture which could attach cell sheet without manual operation by using contracting force of cell sheet. The self-attachable fixture had many hooks aligned in a straight line for suppressing of stress concentration, and was able to attach cell sheet by hooking without piercing cell sheet as multi hook fixture [15]. Since these hooks were semi-fixed against main body of the fixture, hooks were able to touch cell sheet gently, and low damage applied to cell sheet.

Figure 2 shows the Schematic diagram for process of the attaching of cell sheet. First, cells were cultured on polystyrene dish coated with thermo responsive polymer, (N-isopropylacrylamide) (PIPAAm). At 37 °C, the surface of PIPAAm was relatively hydrophobic, but becomes hydrophilic below 32 °C. Below 32 °C, cell sheet spontaneously lifts up from the surface [1-3, 16]. After confirming the formation of cell sheet, cell sheet which was formed dumbbell shape by cell sheet stamp (see II-C) was set on test system (Fig. 2a). Next, self-attachable fixture was set on the cell sheet, and vertical direction force applied cell sheet by tip of hooks. Because the hooks were semi-fixed and could slide vertical direction easily, tip of hooks were touch cell sheet gently. Thus, there was low possibility that the tip of hooks hurt the cell sheet by vertically press force (Fig. 2b). Below 32 °C, culture surface became hydrophilic and cell sheet lifted up (Fig. 2c). Then, cell sheet contracted and curling around the hooks by sytoskeletal tension and reorganization, and attached to the self-attachable fixture (Fig. 2d). After confirming of attaching the cell sheet, self-attachable fixture was lifted and whole of the cell sheet was also lifted from culture surface (Fig. 2e), and finally, tensile test carried out (Fig. 2f). All of this process was performed in culture medium.

The design theories of the self-attachable fixture are discussed below (Fig. 3).

- Single cells with a diameter of 10 μm are ruptured by pressures over 100 nN [17]. Therefore, we assume that the compressive strength of the cells (P_c = 1273 Pa) is obtained as the pressure divided by the total area of the cells.
- When cells are cultured on post arrays, the posts are deflected by traction force of cells. The force which pulls the post is 10 nN [18], and we regard this force as the contracting force of single cells (f_c). The contracting force of the cell sheet (F_c) is obtained by $F_c = f_c \times N_c$ (Eq. 1). Here (N_c) is the number of cells which bind uniformly and align in a straight line vertical to the tensile direction. In this study, the width of the cell sheet was 10 mm, and we assumed that the diameter of the cells was 10 μm. Assuming that the

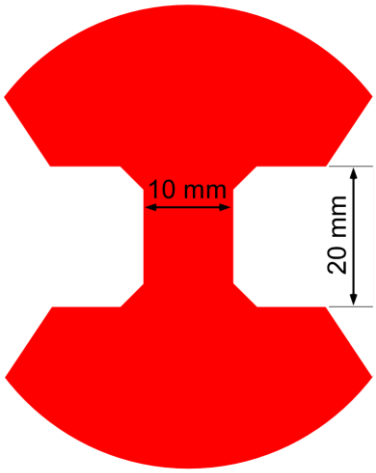

Figure 4. Schematic diagram of prepared cell sheet.

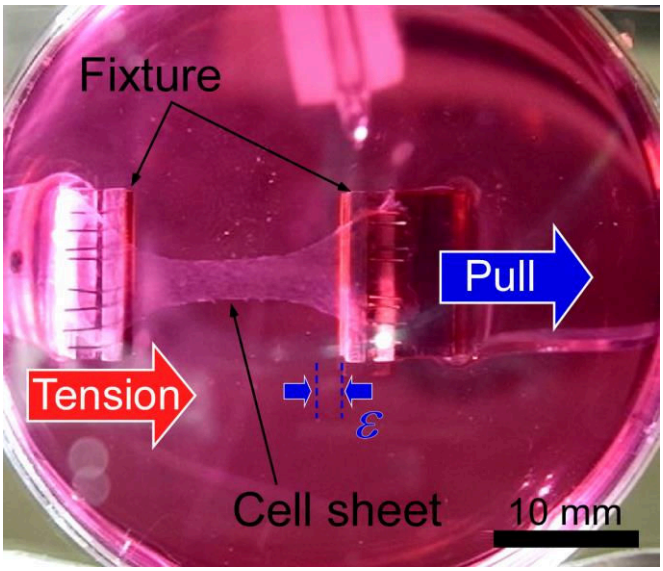

Figure 5. Cell sheet during the tensile test. Both ends of the cell sheet were attached to the self-attachable fixture without piercing and tensile force was applied.

cells bind uniformly and are aligned in a straight line vertical to the tensile direction, we get the number of cells (N_c) as 1000. Inserting these values into Eq. (1), we obtain contracting force (F_c) as about 10 μN.

- When a single cell with a diameter of 10 μm is loaded with 15 % strain, the maximal tension (f_t) is about 0.2 μN [19]. The tension of the cell sheet (F_t) is about 200 μN which is obtained by $F_t = f_t \times N_c$ (Eq. 2).
- In the tensile test, total tension which is loaded on the self-attachable fixture (F_f) is 210 μN, as obtained by $F_f = F_i + F_t$ (Eq. 3).
- The pressure which is loaded on a single cell at tip of one hook (P_h) is obtained by $P_h = F_f/(N_h \times A_h)$ (Eq. 4). Which N_h is the number of hooks and A_h is the area of one hook tip. In this study, we use tungsten wire (0.2 mm diameter tungsten wire, Nilaco, Japan) for hooks. Since tungsten wire has high hardness, hooks were not bent by the tension of the cell sheet. To attach the cell sheet without piercing it, the relationship between P_h and P_c needs to be satisfied $P_h < P_c$ (Eq. 5). After inserting Eq. (4) into Eq. (5), we get $F_f/(N_\mathrm{h} \times A_\mathrm{h}) < P_c$ (Eq. 6).
- Using Eq. (6), we can calculate that the number of hooks (N_h) has to satisfy the condition of $N_\mathrm{h} \geq 8$. Attaching the cell sheet to the tensile test system becomes possible by mechanical hooking with the hooks of the self-attachable fixture. When the density of the hooks (number of hooks: N_h) is increased too much, it is difficult to fix the cell sheet by mechanical hooking. We determined $N_\mathrm{h} = 8$ was the minimum number to attach the cell sheets securely.

C. Preparation of Cells

We cultured C2C12 cells (mouse myoblast cells) in culture medium which contained Dulbecco's modified Eagle medium (D-MEM, High glucose, Nacalai Tesque, Japan) containing 10% fetal bovine serum (FBS, Gibco, CA) and 1 % antibiotic solution (Penicillin-streptomycin solution, Nacalai Tesque). After confirming 80 % confluence, cells were suspended in Dulbecco's Phosphate-Buffered Saline (DMEM, High glucose, Nacalai Tesque) and detached from their culturing dishes by incubating with trypsin solution (TrypLE Express, Gibco, Carlsbad, CA) for 3 minutes. Then, cells were subcultured in culture medium which contained none of FBS. After 5days from subculturing, cells were incubated on a polystyrene dish coated with PIPAAm; other conditions were 5 % CO_2 atmosphere, 37 ˚C temperature for 5 days at a density of 0.5×10^5 cells/cm2.

For the tensile test, cell sheets were compressive cut into dumbbell shapes using a rubber stamp (cell sheet stamp). The cell sheet dimensions were 20 mm (in the tensile direction) and 10 mm (width) (Fig. 4).

F. Protocol of Tensile Test

After getting the dumbbell shaped cell sheets, we set the culture dish into the test system. Then we set the self-attachable fixture on the cell sheet. The cell sheet began peeling off the culture surface by PIPAAm action at 25 °C. Therefore, we had to set the sheet into the system as quickly as possible. About 15 minutes after setting the cell sheet, it had peeled off the culture surface and began to contract and curl around the self-attachable fixture. After confirming sheet attachment, the culture surface was lowered and the whole cell sheet was lifted from the culture surface. To exclude effects of temperature change and air convection, we covered the test system with an acrylic shield. The tensile test was done at 25 °C and the strain speed was 0.5 % per second (0.1 mm/s) (Fig. 5). The tension was measured until it began to decrease. The stress (σ) was given by $\sigma=T/A_0$, where T is the tension measured by a force transducer and A_0 is the initial mean cross-sectional area of the cell sheet. We calculated A_0 by $A_0=t\times w$, where t is cell sheet thickness and w is cell sheet width. The thickness t was assumed as 20 μm [20]. When cell

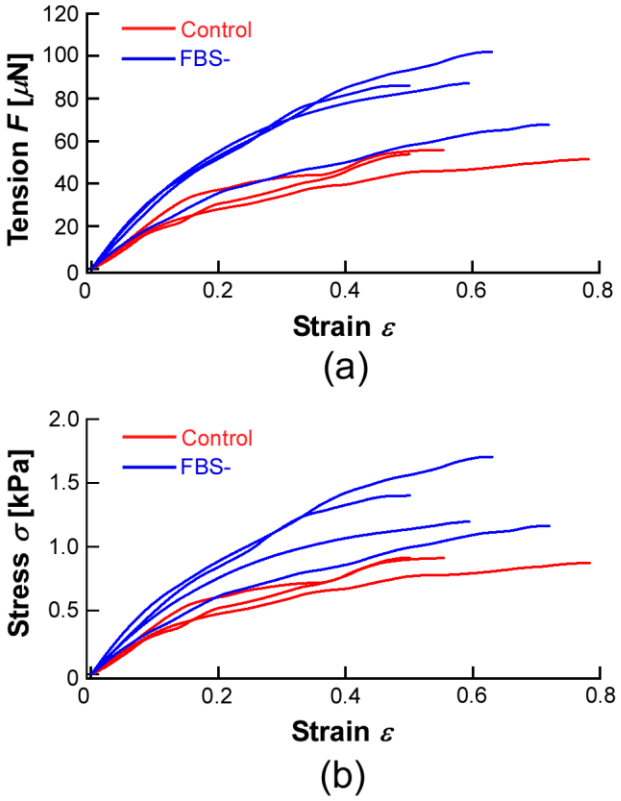

Figure 6. Mechanical properties of the cell sheet (tension and stiffness): (a) tension-strain curves of control and (FBS-); and (b) stress-strain curves of control and (FBS-).

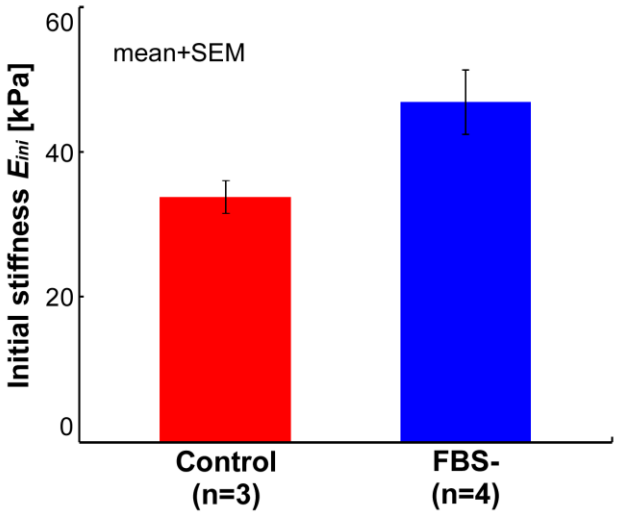

Figure 7. Mechanical properties of cell sheet (initial stiffness). Initial stiffness values of the cell sheets were 33.8 kPa (control) and 46.9 kPa (FBS-).

sheets were detached from the culture surface, they contract and their widths were changed from 10 mm (original width). Thus, we inserted measured value into width w at every test.

The initial stiffness E_{ini} was obtained by fitting a straight line from the origin to the low strain region ($0 \leqq \varepsilon \leqq 0.01$) of the stress - strain curve.

III. Results and Discussions

Fig. 6 shows mechanical properties of controlled cell sheet (control) and cell sheet which subcultured in culture medium which contained none of FBS (FBS-). The self-attachable fixture was able to attach the cell sheets automatically without piercing them. Fig. 6(a) show the tension-strain curves. The ranges of the maximum tension applied to the cell sheets were 51.5 – 55.9 μN (control) and 67.7 – 102.0 μN (FBS-). We found that the maximum strain of cell sheets (strain: 0.50 - 0.78) was smaller than that of single cells (strain: 0.5 - 3) [7, 8] and we considered that the difference was the contribution of initial strain of the cell sheets. In previous studies, single cells detached from the substrate were contracting and so cells were free from initial strain. However, our cell sheets were loaded with the initial strain because that strain occurred when cells adherence to the culture surface was maintained. Therefore, the maximum strain that appeared for the sheets was smaller than that of single cells. Fig. 6(b) show the stress-strain curves. The ranges of the maximum stress applied to the cell sheets were 0.87 – 0.92 kPa (control) and 1.16 – 1.71 kPa (FBS-). Comparing the relationships for tension-strain curve and stress-strain curve, variation of stress-strain curve was more widely than that of tension-strain curve. This was because variation of width was different in each experiment. Fig. 7 shows the Initial stiffness of cell sheet. The initial stiffness values of cell sheets were 33.8 kPa (control) and 46.9 kPa (C2C12). This study represents the first successful measurement of tensile properties of cell sheets. Other conventional tests cannot measure tensile mechanical properties of cell sheets, although tensile mechanical properties of single cells have been measured. We compared the initial stiffness values of cell sheets and those of single cells [7, 8, 21] and judged our present values were reasonable. The initial stiffness of (FBS-) was significantly larger than that of control (P value: 003). Because needed amount of strain for measuring initial stiffness was extremely small (ε = 0.01), there was a little damage when evaluating mechanical properties of cell sheet. By referring to Fig. 7, initial stiffness of (FBS-) was stiffer than controlled. It attributed to effect of differences of differentiation owing to density of FBS. The mechanical properties of C2C12 cells are effected by degree of differentiation [22]. In this study, mechanical properties of C2C12 cell sheet were also effected by differentiations.

IV. Conclusion

In this study, we proposed tensile test for measuring mechanical properties of cell sheet. When applying tensile test, there are difficulties attributed to fragility and contracting of cell sheet. Therefore, we developed self-attachable fixture which can attach cell sheets spontaneously without breaking or tearing them. Because there was no tensile test system with a measurement range that covered the tension range of cell sheets, we also developed a tensile test system. To use developed system for

measurement, we successfully measured mechanical properties (tension, stress and initial stiffness) of cell sheet cultured in controlled culture medium and culture medium which contain none of FBS culture medium (FBS-). The initial stiffness of (FBS-) was significantly larger than that of control (P value: 0.03).

Because we could measure initial stiffness in small amount of strain ($\varepsilon = 0.01$), it is possible that to evaluate mechanical properties of cell sheet with a little damage. Developed system helps to evaluate optimum culture condition for culturing cell sheet which has required stiffness. Thus, we can expect that this study provide fundamental insight to develop cell sheet engineering.

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