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How do chondrocytes aggregate on fibroin substrate*

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Abstract— **The effects of substrate material on the spatio-temporal behavior of cells is an important issue. Although cell aggregation has been observed on various fibroin substrates, the mechanisms of this aggregation have yet to be fully clarified. In this study, cell aggregation behavior on fibroin substrates were evaluated, focusing on the distance between each cell and the direction of individual cell migration. Our results showed that on fibroin substrates cells did not attract each other. However cells stayed close to adjacent cells over 24 hours of cultivation.**

I. Introduction

Cell migration within a three-dimensional matrix and over two-dimensional substrates occurs in a wide variety of physiological and biotechnological situations, such as tissue repair, immune response reactions, and tumor invasion (1). Various stimuli from the surrounding environment are known to affect cell behavior; for example, changes in cell-cell adhesion may initiate cell migration. On the other hand, cell-substrate adhesion also has an important role in regulating cell migration behavior. Hence the effects of substrate mechanics on cell behavior have been under intense investigation.

Fibroin, which is one of the component proteins in silk, and has been widely used in biomedical applications [1–3]. Moreover, in the field of cartilage regeneration, many researchers have investigated its application as a cell scaffold [4,5]. Kawakami et al. used a fibroin sponge as a scaffold for chondrocyte cultivation and demonstrated that initial chondrocyte aggregation led to an enhanced cartilage tissue formation in fibroin sponges [6]. In addition, in a previous study, we investigated cell aggregation behavior on fibroin substrates, and noted that fibroin was able to enhance cell-cell interactions during cultivation and control cell aggregation behavior during cell migration [7]. In general, cell aggregation is one of the key events in cell-cell interaction, making it a vital part of tissue formation. From both scientific and engineering viewpoints, the understanding of the cell-cell adhesion process is important for clarifying and regulating cell aggregation and subsequent tissue formation for various biomaterial (e.g. fibroin).

In this study, cell proximity behavior on fibroin substrates was quantitatively evaluated focusing on two aspects: the distance and the direction of multi-cell movement. Cell-cell distance and its dynamic changes are one of the key factors for characterizing the cell-cell adhesion process. Reinhart-King et al. researched the contribution of matrix mechanics to stable cell-cell contact and suggested that matrix stiffness determined the length over which cells can detect adjacent cells [8]. By understanding the distance in which cell detect surrounding cells, it is possible to gain insights into the cell aggregation mechanism on fibroin. Moreover, cell migration is a multi dimension behavior and its directionality may be another factor in determining cell-cell interaction. In this study, the distance between cells and the direction of cell migrations cultured on fibroin substrates were measured in order to evaluate cell aggregation behavior on fibroin substrates.

II. Materials and Methods

A. Cell preparation

Articular cartilage tissue was aseptically removed from the proximal humerus, distal femur, and proximal tibia of 4-week-old Japanese White rabbits (Oriental Bio Service, Kyoto, Japan). After all adherent connective tissue had been removed, the excised cartilage tissue was diced into 1 mm^3 segments and chondrocytes were isolated by digesting small segments of cartilage with 0.25% trypsin EDTA (Nacalai Tesque, Kyoto, Japan) for 30 minutes in a temperature controlled bath at 37°C. After being rinsed twice with Dulbecco's Phosphate Buffered Saline (PBS; Nacalai Tesque, Kyoto, Japan) and centrifuged at 1500 rpm for 5 minutes, the cartilage was enzymatically digested with 0.25% type II collagenase (CLS-2; Worthington Biochemical, Lakewood, NJ) for 6 hours at 37°C. After straining through a cell strainer (BD Falcon, Franklin Lakes, NJ) and washing twice with PBS, a single cell suspension was obtained. Cartilage harvests from living animals were approved and accepted by the animal care committee of the Institute for Frontier Medical Sciences at Kyoto University.

Cells were passaged once with Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Nacalai Tesque, Kyoto, Japan) and 1% antibiotic mixture (10,000 units/mL penicillin, 10,000 mg/mL streptomycin, and 25 mg/mL amphotericin B; Nacalai Tesque, Kyoto, Japan) beforehand. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 5 days. The medium was changed every 2 days.

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B. Substrates plates preparation

To create fibroin coated plates, an aqueous fibroin solution was prepared as described previously. Briefly, degummed silk fibroin fibers of *Bombyx mori* cocoons were dissolved in 9 M lithium bromide aqueous solution at room temperature, and then the solution was dialyzed against pure water. The concentration of fibroin in the water solution was determined by colorimetric method and was prepared to be 1 wt%. Before coating the fibroin substrate, 35 mm glass bottom dishes (27 mm glass coverslip in diameter; Asahi Techno Grass, Tokyo, Japan) were washed with acetone and completely dried at 50°C. Culture dishes were soaked in fibroin solution for 1 min at room temperature, and then dried at 50°C. The dishes were immersed in 80% methanol solution for 1 hour, and dried again at 50°C.

To create protein coated cell adhesive plates, ProNectin® F (Sanyo Chemical Industries, Kyoto, Japan), which was composed of RGD amino sequences and silk fibroin beta-sheet structures, was prepared according to the manufacturer's instructions. Briefly, stock solution was diluted to 10 μg/mL in PBS at 37°C. Culture dishes were soaked in the diluted solution for 5 min at room temperature. Afterward, the culture dishes were washed twice with PBS.

C. Time-lapse microscopy and cell trajectory acquisition

Passaged chondrocytes were removed from the T flasks by adding 0.25% trypsin EDTA and washed twice with PBS. Soon after, detached cells were suspended in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) containing 10 vol% FBS, 1 vol% antibiotic mixture and 0.2 mM ascorbic acid (A8960; Sigma-Aldrich Japan, Tokyo, Japan). After that, 1.0×10^5 cells were seeded on a dish in cell suspension medium at a concentration of 5.0×10^4 cells/mL (at a density of approximately 1×10^4 cells/cm^2).

Each dish was enclosed in a culture chamber (MI-IBC-IF; Olympus, Tokyo, Japan) in a humidified atmosphere at 37°C and placed on an inverted phase microscope (IX-81; Olympus, Tokyo, Japan). During a 24-hour culture, time-lapse phase contrast images were captured every 10 minutes by a CCD camera (DP70; Olympus, Tokyo, Japan). The image size was 680 × 512 pixels at 1.3 μm resolution.

Every cell captured in time-lapse observation on each substrate (fibroin and ProNectin; $n = 5$ each) was manually tracked using MTrackJ [9], an ImageJ (National Institutes of Health, Bethesda, MD) tracking plugin. Position data of each cell on each frame was measured by the MTrackJ tracking function and was recorded in spreadsheets to calculate distances between each pair of cells and the direction of cell motion.

D. Two cell proximity evaluation

From trajectory data, the Euclidean distance between each pair of cells was measured and the period for which cells remained within a certain distance L was recorded using R (The R Foundation for Statistical Computing, Vienna, Austria). In practice, some cells move into/out of frame during time-lapse observation. Therefore, the Kaplan–Meier estimator was used and out of frame cell data was referred to as censored data. In addition, cell pairs that emerged simultaneously because of frame entrance or mitotic division were excluded in this analysis.

E. Direction of cell migration

In order to assess whether the direction of cell migration enhanced aggregation behavior, a density-based evaluation, which was a modified cell migration analysis method based on Bonnet et al. [10], was used. In short, cell density distributions were evaluated using two-dimensional Kernel density estimation, and subsequently the gradient of the density field was computed. Cell migration direction was measured from cell trajectory data and the relationships between cell migration and density gradient directions were evaluated. In this study, *index* was used to characterize the difference in cell migration direction on each substrate, and an increase/decrease in *index* meant that there were attractive/repulsive movements in the cells' spatio-temporal behavior, respectively (see Appendix A).

F. Cell size measurement

The diameters of round shaped cell were evaluated by using ImageJ. Cells on fibroin substrates were chosen at random and each cell's diameter was measured manually. Assuming that two cells were in direct contact with each other, the distance between them would be the average of their diameters.

G. Statistical tests

The Kaplan–Meier estimator was used to calculate the survival function for cell proximity data for the fibroin and ProNectin groups, and a statistical comparison of survival function was done using the log-rank test. Chi-square tests were used to evaluate heterogeneity of the angle between cell migrations and density gradient directions. The difference between *index* on fibroin and ProNectin was analyzed with Welch's t-test. All tests were performed with a significance level of 0.05.

III. Results

A. Cells maintained rounded shapes on fibroin substrates

Chondrocytes were seeded on each substrate at high density (1×10^4 cells/cm^2) and the distance between each cell and the cell migration directions were evaluated. On the ProNectin substrate, cells elongated and few cells were found to be in contact with each other (data not shown). On the fibroin substrate, however, many chondrocytes maintained a rounded shape and participated in cell aggregation. The size of the round cells on fibroin substrates ranged from 10 to 20 μm in diameter (see Fig. 1).

B. Cells on fibroin remain close to adjacent cells

In Fig. 2, histograms for the distance between each pair of cells on the different substrate are shown. On fibroin substrates, the number of cell pairs peaked when the two cells were located less than 20 μm apart. However, ProNectin substrates did not show this tendency. From this result, we focused on 20 μm because this distance was supposed to be a characteristic distance for cell aggregation behavior on fibroin substrates.

Fig. 3 shows the estimated survival functions of cell proximity-maintaining behavior, which means that cells

remain within 20 μm of an adjacent cell. On fibroin substrates, 31 percent of cell pairs remained close to each other for 1 hour (95% confidence interval, 30-33 percent), while 20 and 15 percent remained close for 2 or 3 hours, respectively. On the other hand on ProNectin substrates, 21 percent of cell pairs remained close to each other for 1 hour (95% confidence interval, 18-25 percent) and no cell proximity maintained more than 15 hours.

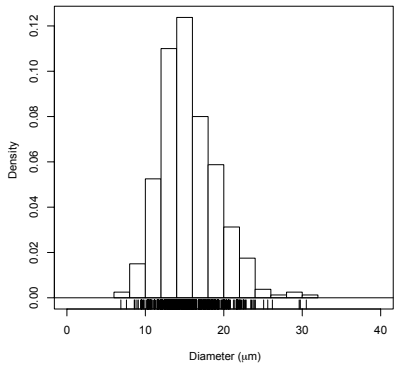

Figure 1. Histogram of the diameters of rounded shape chondrocytes cultured on the fibroin surfaces. N = 401.

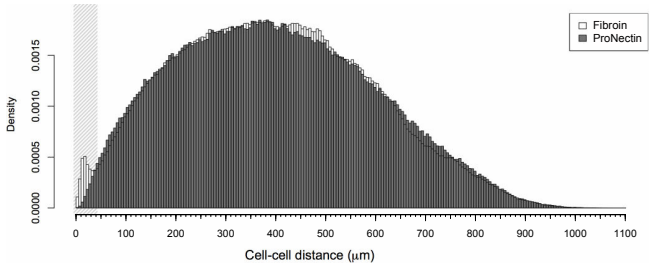

Figure 2. The distribution of computed distance between each pair of cells on the fibroin substrate. A peak was found in cell-cell distance distribution on fibroin, which was not found on ProNectin (the shaded region).

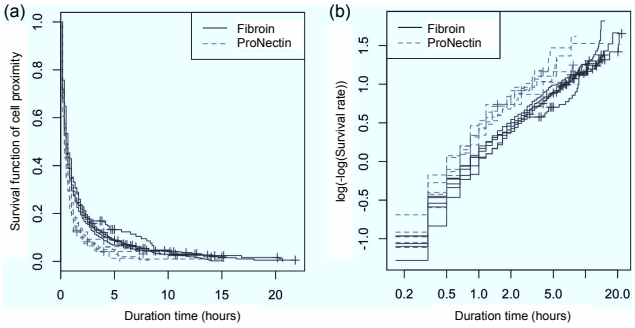

Figure 3. Estimated survival functions (a) and log-log plot (b) of cell proximity-maintaining behavior. Duration time of cell proximity maintainance was significantly different between fibroin and ProNectin substrates (log-rank test; $p < 10^{-6}$). Total sample size was 3855 (including 455 censored data points) for fibroin and 777 (including 39 censored data points) for ProNectin.

C. *Direction of cell migration on fibroin was not biased by cell density*

Fig. 4 shows the distribution of angles between migration direction and cell density gradient on each substrate. Radial axes represent the ratio of actual frequency relative to an ideal frequency distributed evenly over 180 degrees. Therefore, plots with concentric circles indicate that there is no relationship between migration direction and cell density. On ProNectin substrates, 53 percent of cells moved to areas of lower cell density. On the other hand, there were no relationships between cell migration and density gradients on fibroin substrates.

Furthermore, the *index* for ProNectin was smaller than that for fibroin and there was a significant difference between the two groups (Welch's t-test $p<0.005$; data not shown). This difference in the direction of cell migration could be a possible reason for the different cell aggregation behaviors on each substrate.

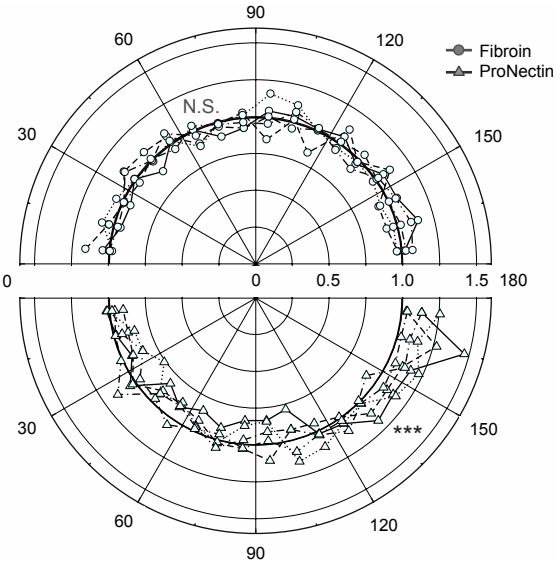

Figure 4. Distribution of the angles θ. The statistical heterogenousity of the angles θ on each substrate was evaluated with Pearson's chi-squared test ($p<0.05$). ***; $p<0.001$. Sample size was 19,995 for fibroin and 16,425 for ProNectin.

IV. Discussions

The purpose of this study was to evaluate the difference in cell aggregation behavior on various substrates. Cell migration on fibroin and ProNectin substrates was observed using phase-contrast microscopy, and the distance between cell pairs and the direction of cell migration were evaluated. The results demonstrated that cell behavior was completely different on fibroin and ProNectin substrates. Cell proximity behavior was observed more frequently and for longer on fibroin substrates compared to ProNectin. However, no attractive behavior was observed on fibroin, whereas cells on ProNectin tended to migrate into areas of lower cell density.

Substrate material and the surrounding environment provide many types of stimuli and influence cell behavior in many ways [11]. For example, Petrie et al. noted that factors such as the topography of the extracellular matrix and receptor signaling promoted directional migration [12]. It is widely said that balance between cell–cell and cell–substrate adhesion is one of the important factors in cell aggregate formation. Moreover, Kambe et al. measured the adhesive force of chondrocytes on fibroin and ProNectin, and discussed the possibility of the substrates' effects on chondrocyte's phenotypes [13]. Taking the above into

consideration, low cell-substratum adhesiveness probably led to the stable cell proximity behaviors seen on fibroin substrates in this study.

Abercrombie et al. proposed the existence of contact inhibition of locomotion (CIL), in which a migrating cell in contact with another migrating cell changes direction to move away from the point of contact [14,15]. This mechanism is still not fully understood. However, it is generally said that cell-substratum adhesiveness is one of the possible causes for cell protrusions and migrations [16]. From this, cell density dependent migration observed in this study could be caused by adhesion provided on ProNectin substrates. Moreover, the fibroin surface might suppress CIL, influencing cell aggregation formation.

V. Conclusion

We performed two types of evaluation for different cell aggregation behavior. The results showed that cell proximity behavior was observed more frequently and for longer on fibroin than on ProNectin. However, no attractive behavior was observed in cell aggregation on fibroin, whereas cells on ProNectin tended to migrate into areas of lower cell density.

Appendix A: Cell Migration Analysis

The relationship between the direction of cell migration and the gradient of cell density was evaluated using the following procedure.

- The direction of cell migration $\boldsymbol{v}_i(t)$ was computed for the different images at a time of t and $t+\Delta t$.
- A potential field based on the local density of cells is computed according to the two-dimensional Kernel density estimation: the density of cells at position $\boldsymbol{x}$ is computed as a function of the positions of cells $\boldsymbol{x}_i$, according to

$$f(\boldsymbol{x},t)=\frac{1}{nh^2}\sum_i^n \mathrm{K}\left(\frac{\boldsymbol{x}-\boldsymbol{x}_i}{h}\right) \tag{A1}$$

  where n is the total cell number, h is the bandwidth and K is the kernel function. Gaussian kernel was used in this study.
- The gradient of the cell density field $\boldsymbol{n}(\boldsymbol{x},\mathrm{t})$ at position $\boldsymbol{x}$ in each frame t was computed. After $\boldsymbol{v}_i(\mathrm{t})$ and $\boldsymbol{n}(\boldsymbol{x}_i,\mathrm{t})$ were calculated, the correlation of these two vectors was calculated for each cell i, as follows.

$$\cos\theta=\frac{\boldsymbol{v}\cdot\boldsymbol{n}}{|\boldsymbol{v}||\boldsymbol{n}|} \tag{A2}$$

- In order to evaluate the tendencies of cell density-based migration, weighted average of $\cos\theta$ values over the total number of cells and frames was calculated. This *index* ranges from -1 to 1 as its value is increased or decreased by attractive or repulsive behavior in the cell population, respectively.

$$index=\frac{\sum_{i,t}\left(\left|\boldsymbol{n}(\boldsymbol{x}_{i,t},t)\right|\cos\theta_{i,t}\right)}{\sum_{i,t}\left|\boldsymbol{n}(\boldsymbol{x}_{i,t},t)\right|} \tag{A3}$$

As is customary, cells located in border zones of the image were excluded from this migration analysis, as no correct density information relating to their final distribution can be taken from the image. The bandwidth h was 51 μm in this study.

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