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Tensile behaviour of fibroblasts cultured in collagen gel

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An *in vitro* morphological study on the relation of the stress field and the orientation of cells with the use of the time lapse video recording system was carried out. Specimens of thin collagen gel membrane, within which cells are proliferating, were examined under various mechanical conditions. Cell stretching, cell orientation, cell migration, cell proliferation and generation of tensile stress in collagen gel were recorded. It was demonstrated that fibroblasts generate tension and change their orientation along the tensile direction. A hypothetical mechanism for such phenomena is proposed, that is, fibroblasts generate tension and make tense collagen fibres, then cells stretch themselves along the tense fibres and increase tension in this direction. Thus, this mechanism works as a positive feedback loop which enables cells to make tensile stresses and align along them in accordance with the mechanical environment. © 1996 Elsevier Science Limited

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Connective tissues in our bodies carry out functions as mechanical components. These tissues remodel themselves to adapt to the mechanical environment. They undergo hypertrophy if they are subjected to excessive load, and atrophy to reduced load[1]. Hence the dominant cell population within them, fibroblasts in the case of fibrous tissues, should recognize and respond to the mechanical environment around them. Actually, mechanical responses of cells have been observed in culture systems, as the contraction of collagen gel within which fibroblasts are proliferating[2], and the orientation of fibroblasts along the lines connecting two particles to which collagen fibres are attached[3–5]. These observations showed that fibroblasts express mechanical functions, and they could be investigated in detail with the use of an *in vitro* model. Unfortunately, the *in vitro* studies concerning this topic, until now, do not show the mechanical point of view clearly. Hence we have been developing an experimental model in which fibroblasts are cultured in thin collagen gel membrane[6]. This model has proved to be quite convenient for mechanical manipulations and microscopic observations. One of the conclusions obtained in our previous studies using this model is that the response of cells is a dynamic one. In some cases they complete the process within an hour, although the precise course of the process cannot be clarified due to technical difficulties. In this paper, we investigated the dynamic response of cultured cells with the use of the time lapse video recording system.

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MATERIALS AND METHODS

Cell culture

Primary fibroblast-like cells were obtained by the explant method from the synovial membrane of the knee joints of Japanese white rabbits. Primary cells and subsequent cultures were maintained in αMEM (with nucleosides, Gibco, Tokyo) supplemented with 10% calf serum and 60 $\mu g\,ml^{-1}$ Kanamycin. First to fifth cultures were used for the following experiments.

Specimens

The typical specimen used is illustrated in *Figure 1*. The thin collagen gel membrane of $10 \times 10\,mm^2$ is supported by a stainless steel wire mesh (#80) which has a $5 \times 5\,mm^2$ square opening. The specimen was made by pouring the mixture of 8 parts of collagen solution (CELLMATRIX type I-A, Nitta Gelatin, Osaka), 1 part of ×10 MEM (Nissui, Tokyo) solution, and 1 part of buffer (4.77 g HEPES/100 ml 0.08 N NaOH solution) into a silicone rubber mould on which steel mesh had previously been set, and keeping it in a 37°C incubator for 15 min for gelation. The thickness of the specimen was about 0.5 mm, although it could not be set exactly since the upper surface of the mould was left open.

Distribution of cells within specimens

In the case of a specimen with uniformly dispersed cells, cells were dispersed in the neutralized collagen solution before gelation. On the contrary, in the case of

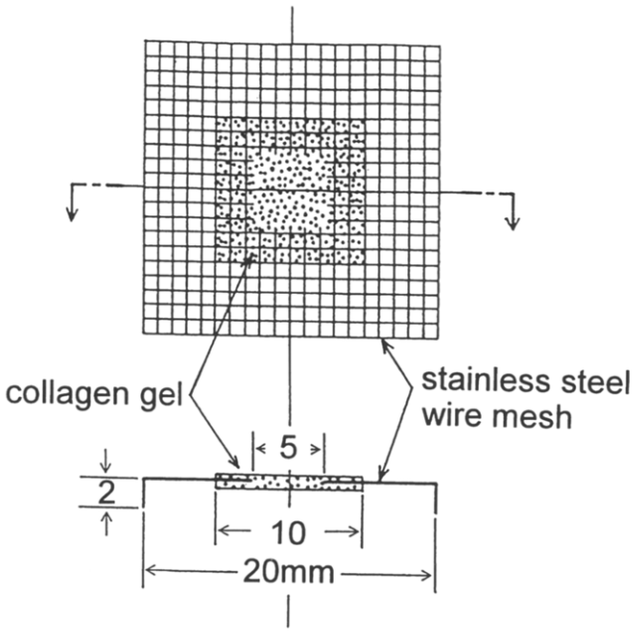


Figure 1 Schematic diagram of the specimen.

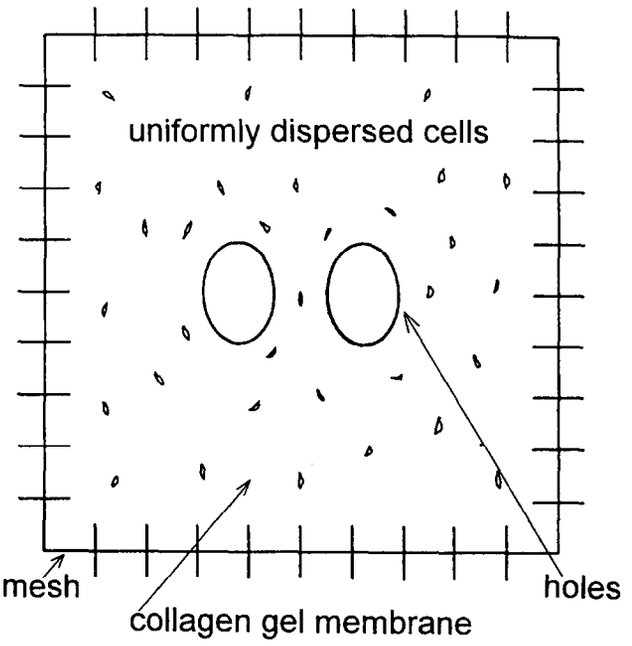


Figure 2 Scheme of Experiment 1.

a specimen with non-uniformly dispersed cells, a mass of cells with size of *ca* 1 mm^3 was attached on the stainless steel mesh near the edge of the opening just before pouring the neutralized collagen solution. Outgrown cells proliferated and migrated from the mass, and a non-uniform distribution of cells was realized.

Mechanical boundary condition of the specimens

The displacement boundary conditions (rigid support conditions) were realized on the boundaries of the specimens where they were supported by the stainless steel wire mesh. The stress free boundary conditions (traction free conditions) were realized on the free boundaries of the specimens where a cut was introduced in the specimens by a surgical knife or where a hole was made at the preparation of the specimens.

Observation of specimens

After gelation, the specimens were placed in plastic Petri dishes (60 mm diameter, Iwaki, Tokyo) filled with medium and kept in a 37°C, 5% CO_2 incubator. The medium was changed every 3 or 4 d. The specimens were observed by a phase-contrast microscope (DIAPHOTO TMD, Nikon, Tokyo) equipped with an incubating box. The specimens were inspected every day and time lapse videos were recorded at occasions of interest.

RESULTS

Experiment 1

By introducing bubbles within collagen solution in the preparation of the specimens, we were able to make holes in collagen membrane and thus make a bridge of collagen gel, as illustrated in *Figure 2*. The specimen had an original cell density of 2×10^6 ml^{-1}, and was incubated several days before the observation. Some selected photos from the time lapse video recordings are shown in *Figure 3*. The time indicated in the photos was measured from the beginning of the recording. The bridge was pulled by both ends, stretched and then torn off spontaneously. The whole process was completed in 10 h. However, the specimens in which we did not disperse cells in collagen gel did not show any deformation at all (data not shown). The fact that cultured cells generate tensile stress in collagen gel can be confirmed visually in these experiments.

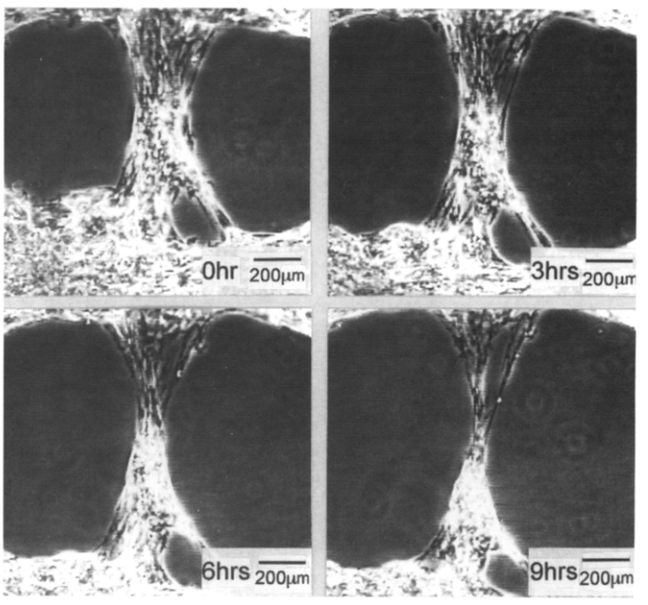


Figure 3 Generation of tensile stresses by cells.

Experiment 2

With cells proliferating and migrating from a mass of cells, and introduction of cuts in the membrane of gel along both sides, as shown in *Figure 4*, we can obtain the specimens with cells proliferating in the tensile stress field. In fact, this geometry of the specimens realizes the uniaxial tensile stress field generated in the collagen membrane. This tensile stress is in the vertical direction in *Figure 4*. At the time when frontal cells had reached the central portion of the specimen, the behaviour of the cells was recorded by the time lapse video system. Typical behaviour of the cells is shown in *Figure 5*. The time indicated in the photos was measured from the beginning of the recording. The cells were apparently lying on the membrane of collagen gel. Most of the cells took the elongated form in the tensile direction. Cells took the rounded form just before mitosis, and then daughter cells recovered elongated shapes immediately. This re-elongation process was very rapid and it appears that cells know the direction in which they would elongate. The cell migrations were also observed and these cells took elongated shapes too.

Experiment 3

Just as in Experiment 2, the specimens with cells proliferating in the tensile stress field were utilized. In this experiment, however, a cut was introduced after the cell orientation was realized, as shown in *Figure 6*. Since this manipulation release the residual stresses developed in the specimen, the cells would experience a change of stress field, and would exhibit the responses against it. Before the experiment, the cells took the elongated shape in the vertical direction, as shown in *Figure 6a*. At this time, we introduced a cut in the central portion of the specimen in a horizontal direction, as shown in *Figure 6b*. The time lapse video was then recorded to observe the behaviour of cells just above the cut. Some typical photos are shown in *Figure 7*. The time indicated in the photos was measured from the instance of the knife cut. About 10 min after the introduction of the cut, the cells took the rounded forms. Then as time proceeded, they took the elongated forms again but in a horizontal direction (*Figure 7*). The cells elongated immediately as if they knew the direction in which to elongate, although the details were not clear using our phase-contrast microscopy. The phenomena had completed within an hour. It should be noted that when the number of cells was small in specimens, the reorientation phenomena described above could not be observed (data not shown).

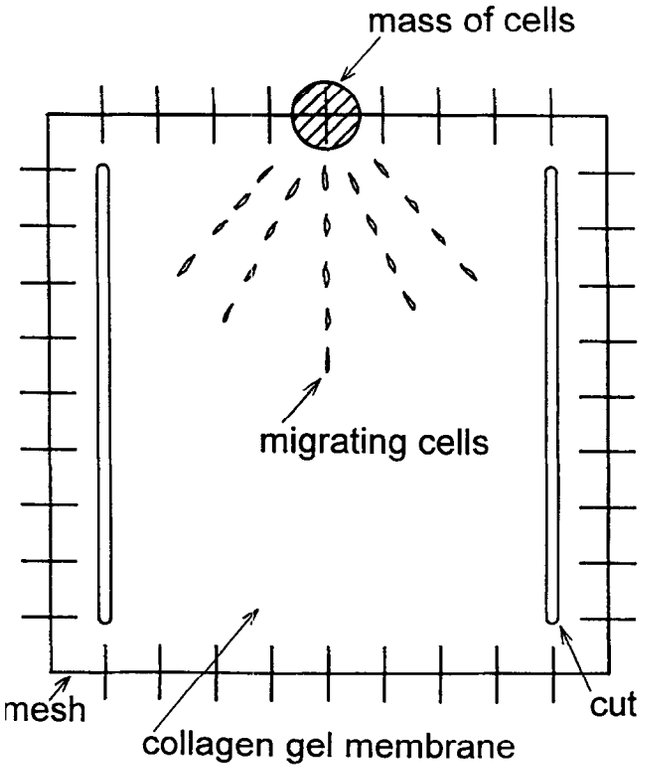


Figure 4 Scheme of Experiment 2.

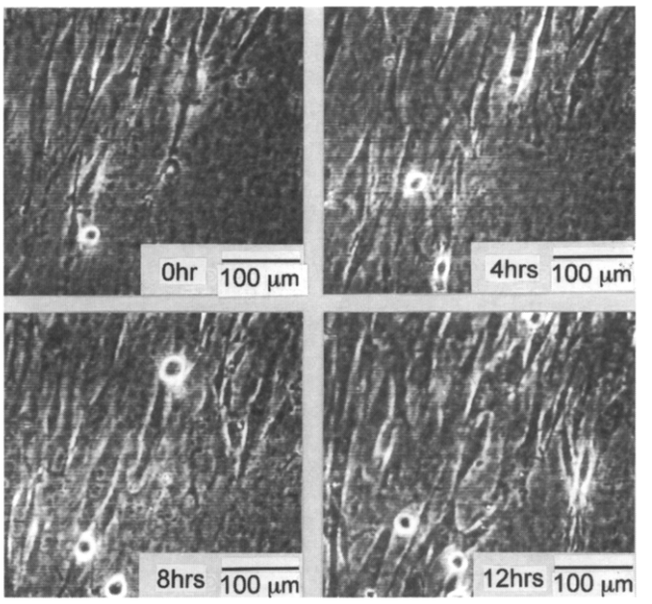


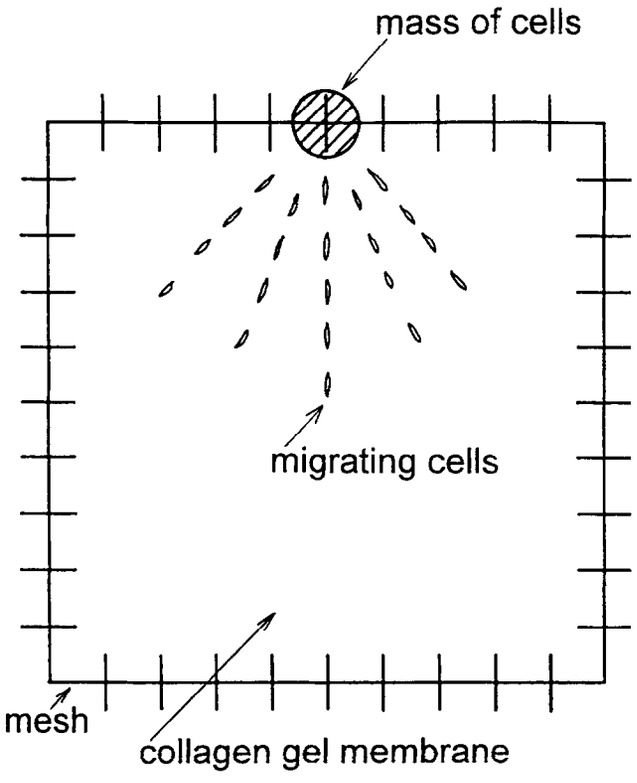
Figure 5 Cell orientation along the tensile stress direction.

DISCUSSION

Generation of tensile stresses by fibroblasts

It is well known that if a mass of collagen gel containing fibroblasts within it is freely drifting in the medium, it will contract as cells proliferate[2]. This contraction is believed to be due to the function of the cells, i.e. the exertion of contracting forces. If the periphery of the mass of gel is attached to surrounding tissues or materials, the situation which may arise most frequently *in vivo*, tensile stresses should be generated in collagen gel as cells contract. Although these stresses cannot be observed directly, their existence can be demonstrated by the appropriate mechanical manipulations[6]. The result of Experiment 1 is one such example, in which the membrane of collagen gel was fixed to the metal mesh along its periphery. The introduction of bubbles in the membrane made a bridge of collagen gel, whose lateral sides were free

from stress, and thus the stress within the bridge was restricted to be uniaxial. As cells contracted the bridge was pulled by both ends and stretched, and the tensile stress was generated in the bridge. The fact that the tensile stress was generated in the gel is clearly illustrated in the above-mentioned photos.



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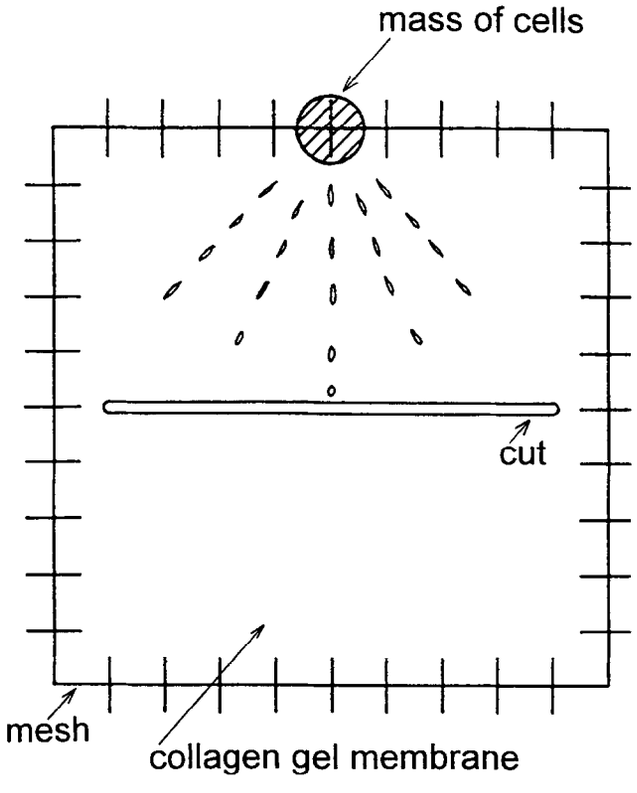

b

Figure 6 Scheme of Experiment 3 before (**a**) and after (**b**) the introduction of the cut.

Cell orientation along the tensile stress direction

In Experiment 2, it was demonstrated that cells stretch themselves along the tensile stress direction. This means that the cells recognized some physical quantities relating to tension. Since it is very difficult for the cells to recognize the stress itself, we believe that they recognize and respond to the nature of collagen fibres they are attached to. As is well known, the cells can stretch along the collagen fibres they are attached to by the contact guidance. Hence if randomly orientated cells exert contraction force between their focal contacts, and if the direction of tensile stress is restricted as in the region near the free surface of the gel, the collagen fibres are stretched and change their orientation to align in the parallel direction to the free surface of the specimen. The cells attached to these fibres would exert force in this direction. Thus, the positive feedback loop between the orientation of tension and that of collagen fibres is realized, and the randomly orientated cells in the beginning will align themselves in the tensile direction as they proliferate. The fact that the cells, just after the mitosis, took elongated forms so quickly may be explained by the existence of well developed orientation of collagen fibres in the gel.

In Experiment 3, the cells must be attached to the collagen fibres before the introduction of the cut, since they exhibit elongated forms. After cutting, the collagen fibres lost tension. This may correspond to the observation that the cells took the rounded form after the cutting. It appears that the cells cannot keep their elongated form if collagen fibres have lost tension. The cells seemed to lose adhesion to the fibres

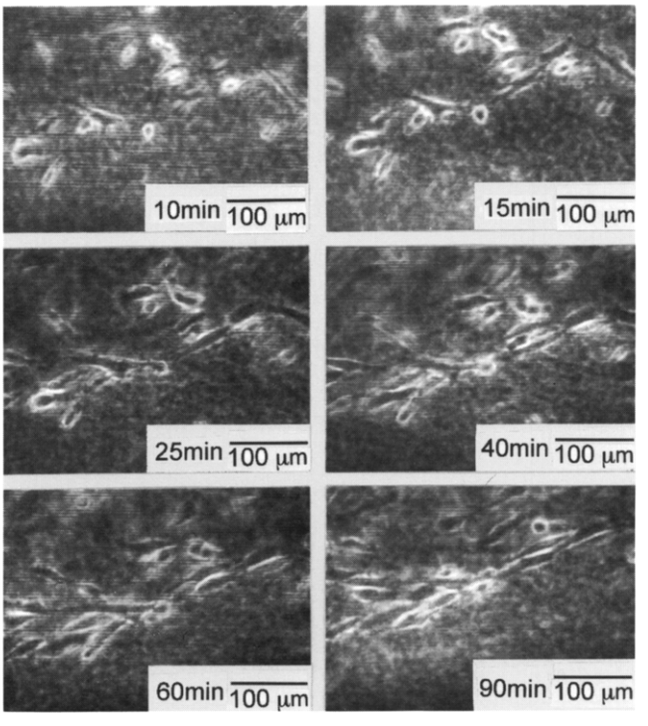

Figure 7 Reorientation of cells toward the tensile stress direction.

previously attached, and then began to attach to other fibres. These fibres lay parallel to the surface of the cut and possibly stretched in this direction. The fact that this process takes only an hour and the cells do not seem to look for the directions to elongate suggests that the cells recognize the tense collagen fibres. We believe that the cells require some anchors to attach to and stretch themselves over them. The rigid anchors are required, and in the case of the soft materials, such as the collagen fibres in these experiments, they must be in tension to be rigid enough for the attached cells. Although interactions between the cytoskeleton and extracellular collagen fibres are believed to be responsible for these phenomena, the details of the process cannot be elucidated in this observation. Further investigations are required to understand how the interaction mechanism works.

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