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Stretching and movement of fibroblasts and osteoblasts cultured in microchannel and micropit arrays

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ABSTRACT

Tissue cells bind to extracellular matrix (ECM), and this attachment to ECM plays an essential role in their growth, function, and even survival. Furthermore, the geometry of ECM is known to play an additional role in regulation for these cells to proliferate and differentiate so that tissues with normal morphologies can be formed or maintained. We attempted to culture fibroblast, osteoblast, and bone marrow derived cells in previously described microchannel arrays and newly created micropit arrays, both coated with ECM protein collagen, to examine usefulness of microfabricated structures for elucidating "what is geometry?" for cells. Cells were inseminated in the well in front of a microchannel array and their movement and stretching behavior against the microchannel array including the entrance and exit terraces were observed using a microscope-TV camera-time lapse video recorder system for 24 hours. Cells entered into the entrance terrace and showed active motions including extending pseudopodia into the channels and whole cell passage through the channels, and fully stretched in the entrance terrace in 8 hours or so. Those cells, however, voluntarily detached themselves from the area in another 8 hours or so probably because of worsening condition of nutrient supply there. Micropit arrays used in the present study consist of a regular arrangement of circular or square pits of diameter or side length of 25, 50, 100, 200, 400, and 600 μm and depth of 10 μm with one size per array or chip. The total area of the pits was designed to be equal to the rest surface area. After four days of incubation of bone marrow derived cells, the total number of cells and the number of cells in the pits were counted. The former and the ratio of the latter to the former appeared to become maximal when the pits of diameter or side length of 100 and 50 μm were used, respectively.

Keywords: microchannel array, micropit array, extracellular matrix, cell culture, fibroblast, osteoblast

1. INTRODUCTION

Previous reports¹⁻⁵ have described usefulness of microfabricated channel arrays for studies of rheologic or deformability properties of blood cells. Microchannels served as a model of physiologic capillaries and, because of their dimensional accuracy and visual accessibility, gave a significant advantage for quantifying the factors dominating capillary blood flow. We could also show changes in the cell wall stiffness of yeast cells during cell cycle by forcing them to pass through the microchannels.⁶ These cells are free-floating cells. Meanwhile, cells that form tissues bind to extracellular matrix (ECM), and this attachment to ECM plays an essential role in their growth, function, and even survival. Furthermore, the geometry of ECM is known to play an additional role in regulation for these cells to proliferate and differentiate so that tissues with normal morphologies can be formed or maintained.

So far, studies have shown the importance of ECM geometry particularly with osteogenesis; bone is constantly remodeled and it appears that osteoblasts recognize pits in bone matrix created by osteoclasts before proceeding to osteogenesis. Clinically, anomalous proliferation and differentiation of fibroblasts or osteoblasts cause heterotopic ossification when they enter into microcracks in other tissues such as ligaments. Allogeneic implantation of powders of demineralized collagenous bone matrix has been used to study the effect of geometry or size on the induction of bone.^{7,8} Coarse powders induced bone whereas fine powders did not. One of the present authors (Kuboki, Y.) and others^{9,10} have used porous hydroxyapatite particles and blocks with bone morphogenetic protein (BMP) and found that

300-400 μm pores were most effective in inducing attachment, differentiation and growth of osteoblasts and vascularization. Recent studies^{11,12} have introduced the photolithographic processes into in vitro cell culture to accurately define cell binding areas on a substrate. Silicone microgrooved surfaces, which were replicas of microgrooved silicon surfaces, were used to study orientation of ECM protein deposition, fibroblast cytoskeleton, and attachment complex components on such textured surfaces.¹³

The aim of the present study is to further examine usefulness of microfabricated structures for elucidating "what is geometry?" for cells. We attempted to culture fibroblast, osteoblast, and bone marrow derived cells in the microchannel arrays and newly created micropit arrays, both coated with ECM protein collagen.

2. MATERIALS AND METHODS

a) Microchannel arrays

Microchannel arrays used in the present study are those previously described in detail.^{5,6} Their principal structure is shown in Fig. 1. In this structure, microgrooves formed in the (100) surface of a single-crystal silicon substrate are converted to leak-proof microchannels by tightly covering them with an optically-flat glass plate. The contact between the silicon substrate and glass plate can be made water-tight by mechanical pressing alone because of optical flatness of both surfaces. This structure makes it possible to microscopically observe movement and stretching behavior of cells through the entire microchannel and also for channels to be cleaned fully for reuse by detaching the two substrates from each other. The terrace structure increased visibility of cells under reflecting illumination.

Dimensions and number of microchannels (microgrooves) used are 6, 7, and 8 μm in width, 20, 30, and 40 μm in length, respectively, and 4.5 μm in depth, and 4704 in parallel. The width of the entrance and exit terraces is 30 μm . The size of each chip is 12 x 12 mm. These chips are now commercially available as "Bloody 5 chips" from Hitachi Hiramachi Electronics Co. Ltd. (Fax: +81-294-23-1350).

b) Micropit arrays

Circular and square pits of diameter or side length of 25, 50, 100, 200, 400, 600 μm and depth of 10 μm were created in the (100) surface of a single-crystal silicon wafer with one size per chip area (12 x 12 mm) by dry-etching to 8 μm depth followed by wet-etching for the rest 2 μm . The total area of the pits was designed to be equal to the rest surface area.

c) Coating with ECM collagen

A fully cleaned microchannel array chip was set in the holder in a clean bench and sterilized by infusion of 80 % ethanol through tubes connected to the holder. After replacing the filling liquid with autoclaved phosphate buffered saline (PBS), the holder was immersed in ice-cold water in a jar, and the filling PBS was further replaced with ice-cold 0.1 M acetic acid and, then, ice-cold 0.1 M acetic acid containing 0.1 % collagen (cell-matrix 1-A type). After keeping the exposure to 0.1 % collagen for 30 minutes, the filling solution was fully replaced with PBS. Finally, the holder was filled with a culture medium (Minimum Essential Medium Alpha Medium with 10 % fetal bovine serum (FBS); GIBCO BRL) and warmed to 37 C in an incubator. Micropit array chips were similarly coated with collagen using autoclavable capped plastic beakers.

d) Cell preparations

Frozen fibroblast strain cells (3T3-L1) and osteoblast strain cells (MC3T3-E1) were thawed, washed with the culture medium and incubated in the medium using culture dishes in a CO₂ incubator (5 % CO₂, 37 C). When cells grew to become 80 % confluent, they were detached from the dishes by treatment with trypsin-EDTA solution (Sigma 1 x solution; 2 min) and suspended in the medium at a concentration of about 1×10^4 cells/ml.

Bone marrow stromal cells were obtained from femurs of anesthetized Wistar rats (male, 6 weeks) and incubated in dishes. Cells were harvested after growing to 80 % confluent state and suspended in the medium similarly.

e) Cell incubation in microchannel arrays

An aliquot (200 μl) of the cell suspension was gently introduced through the inlet hole of the holder into the well in front of a microchannel array using a micropipette or 1 ml disposable syringe. The holder was covered with aluminum foil and placed on the stage of an inverted microscope that was set in an incubator (37 C). Cell behavior was observed and recorded by a TV-camera attached to the microscope and a TV-monitor and time-lapse video recorder outside the incubator.

f) Cell incubation in micropit arrays

Two micropit array chips were placed in each of culture dishes. Two ml cell suspension was taken into each dish

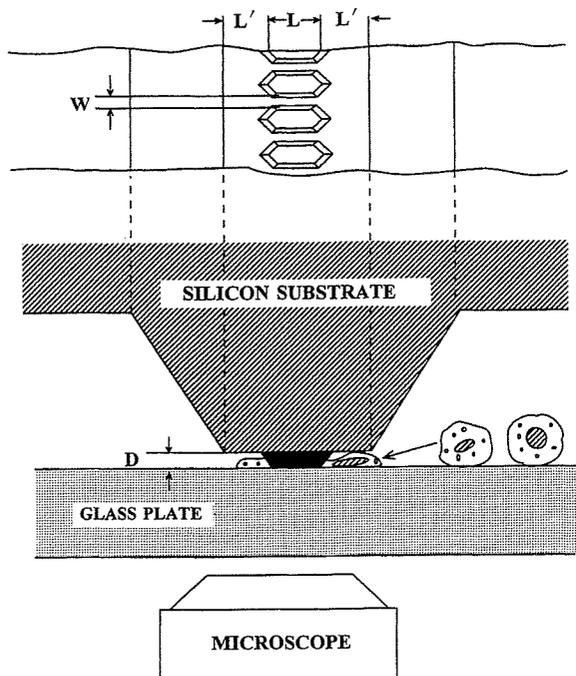


Fig. 1. Outline structure of microchannels formed between the surfaces of a single-crystal silicon substrate and a glass plate.

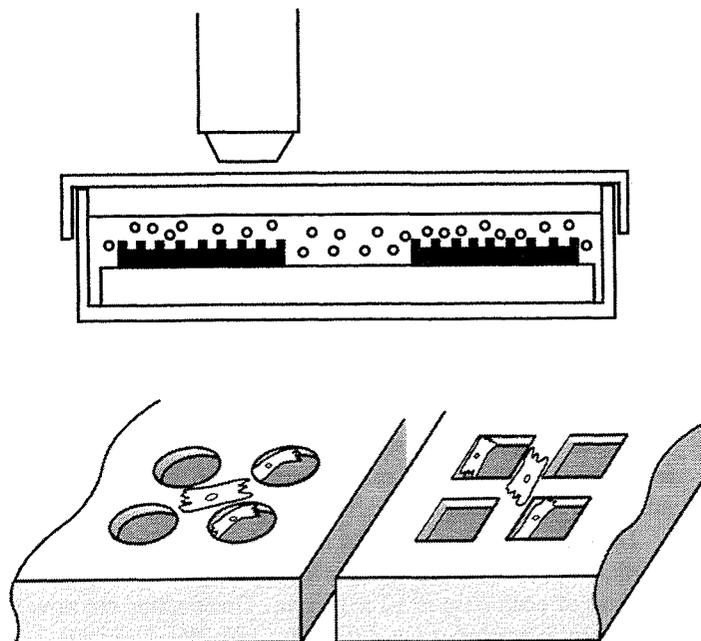


Fig. 2. Schematic drawing of cell culture in micropit arrays.

and incubated in a CO₂ incubator (5 % CO₂, 37 C). The setting is schematically shown in Fig. 2. The spacer disk was used for microscopic observation of cells over the cap. The medium was changed to fresh one on day 2 of incubation and cells attached inside and outside the pits were microscopically counted on day 4 of incubation. Then, the chips were gently flushed with PBS and cells were detached from each chip by trypsin-EDTA (1x solution, 1ml; Sigma) treatment and transferred into 4 ml medium together with the trypsin solution and collected by centrifugation (1000 rpm, 5 min). The cells were resuspended in 100 μl medium and cell number in the suspension was counted using a Thoma cell counter. In another run, the incubation of cells was continued up to 29 days with the culture medium changed every 3 days.

RESULTS

a) Cells cultured in microchannel arrays

Pictures obtained at 3.5 hours and 5.5 hours after the start of incubation of osteoblast cells are shown in Fig. 3. Cells appeared to voluntarily enter into the entrance terrace, extending pseudopodia there. A few cells further passed through the microchannels (3.5 h). The number of cells that passed through the microchannels increased considerably in another two hours (5.5 h).

Figure 4 shows pictures of fibroblast cells taken at 10 min, 1, 4, 8, 12, and 16 hours after the start of incubation. The fibroblast cells appeared to be slightly larger than the osteoblast cells in Fig. 3 and fewer cells passed through the microchannels. Cells fully stretched instead in the entrance terrace area in 8 hours or so. However, those cells voluntarily detached themselves from there in another 8 hours or so.

b) Cells incubated in micropit arrays

Figure 5 shows pictures of bone marrow derived cells incubated for 9 days in different size micropit arrays. As indicated by the pictures, a higher tendency was observed for cells to exist inside the pits than outside the pits. Figure 6 shows the total number of cells obtained by the two counting methods. The closed bars are counts by microscopic counting whereas open bars are counts of cells detached from the chips. It was difficult to microscopically count

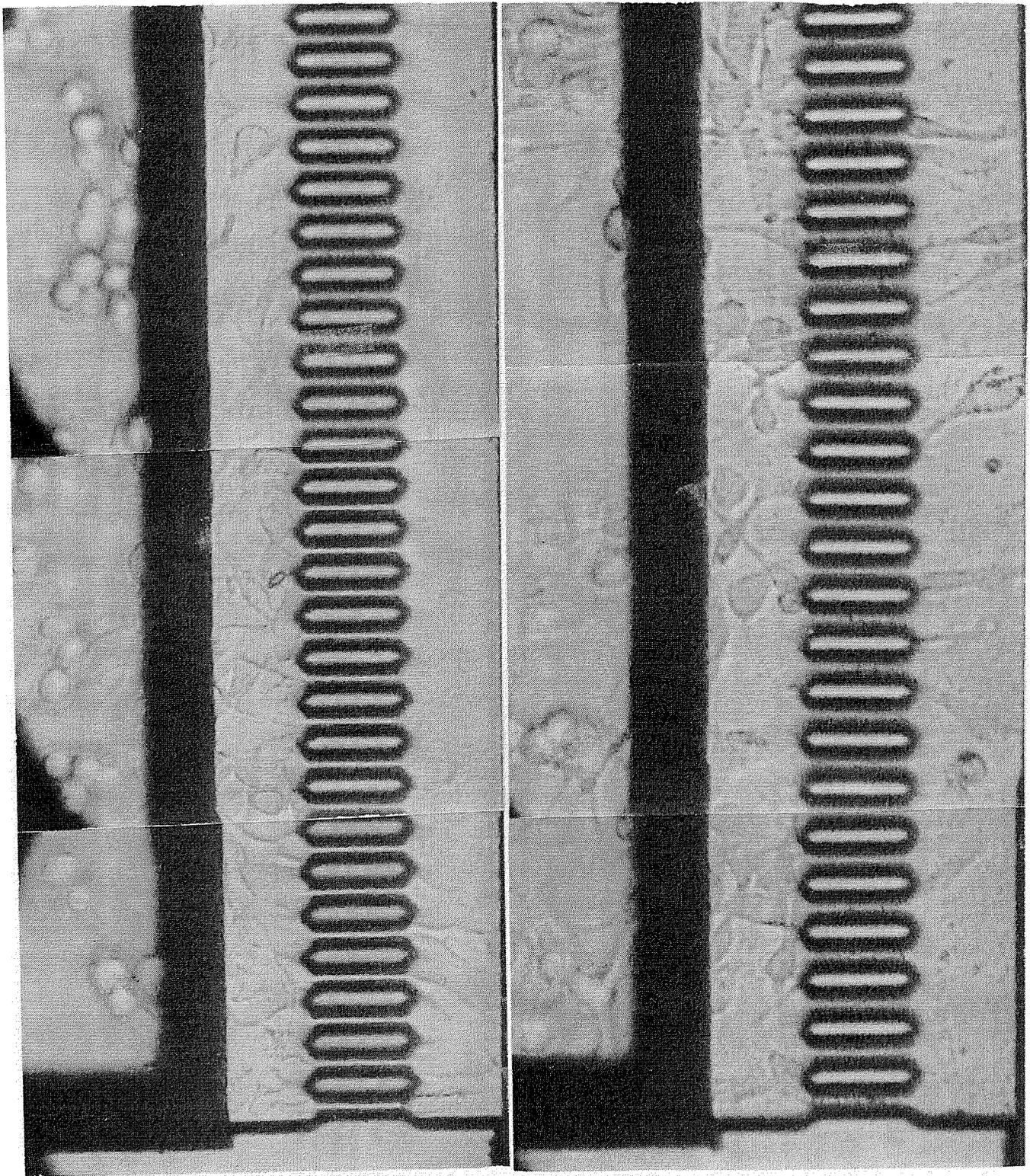


Fig. 3. Pictures of osteoblast strain cells taken at 3.5 hours (left panel) and 5.5 hours (right panel) after the start of incubation of cells in a microchannel array (channel width 8 μm , length 40 μm).

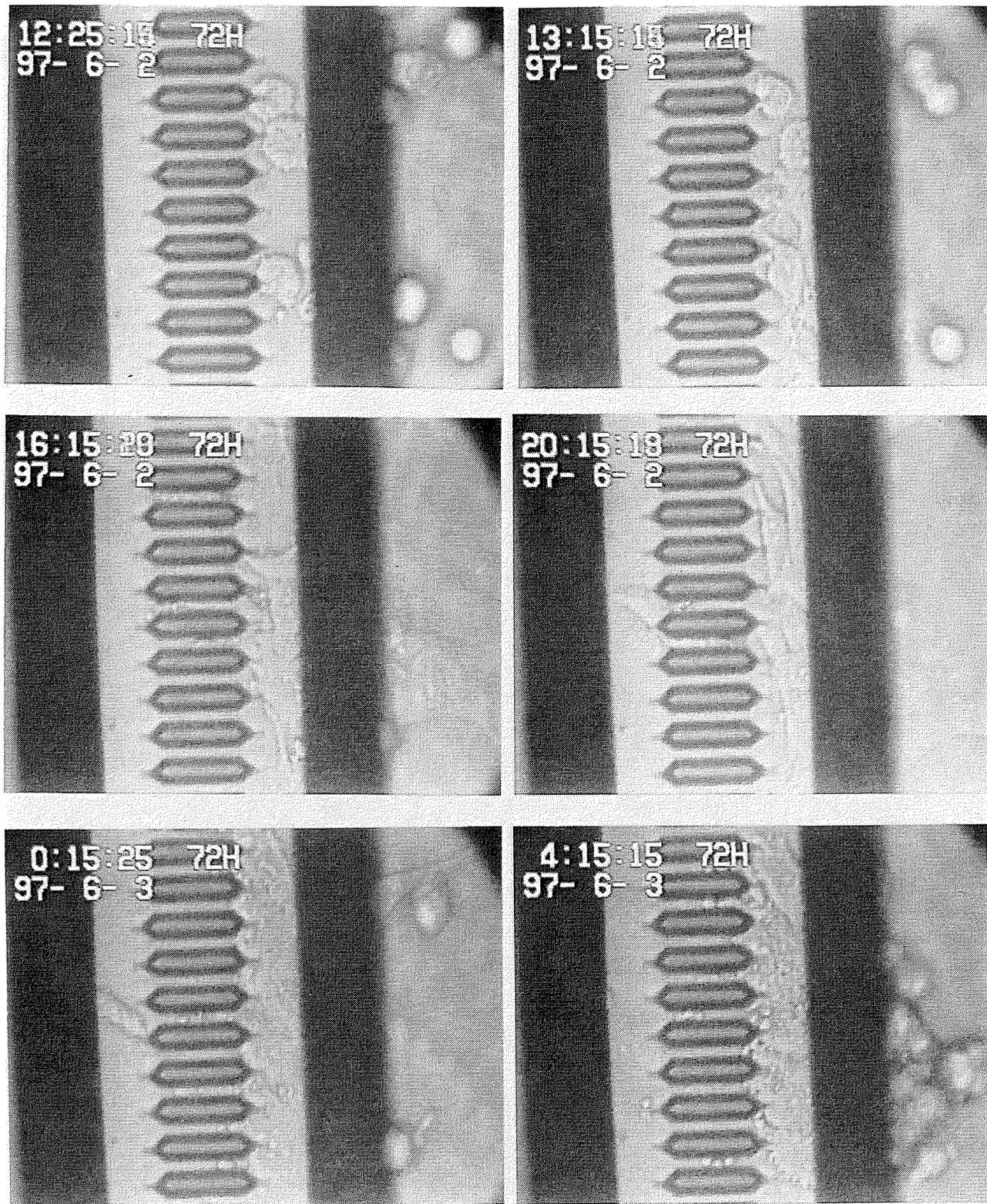


Fig. 4. Pictures of fibroblast strain cells taken at 10 min (upper left), 1 (upper right), 4 (middle left), 8 (middle right), 12 (lower left), and 16 hours (lower right) after the start of incubation of cells in a microchannel array.

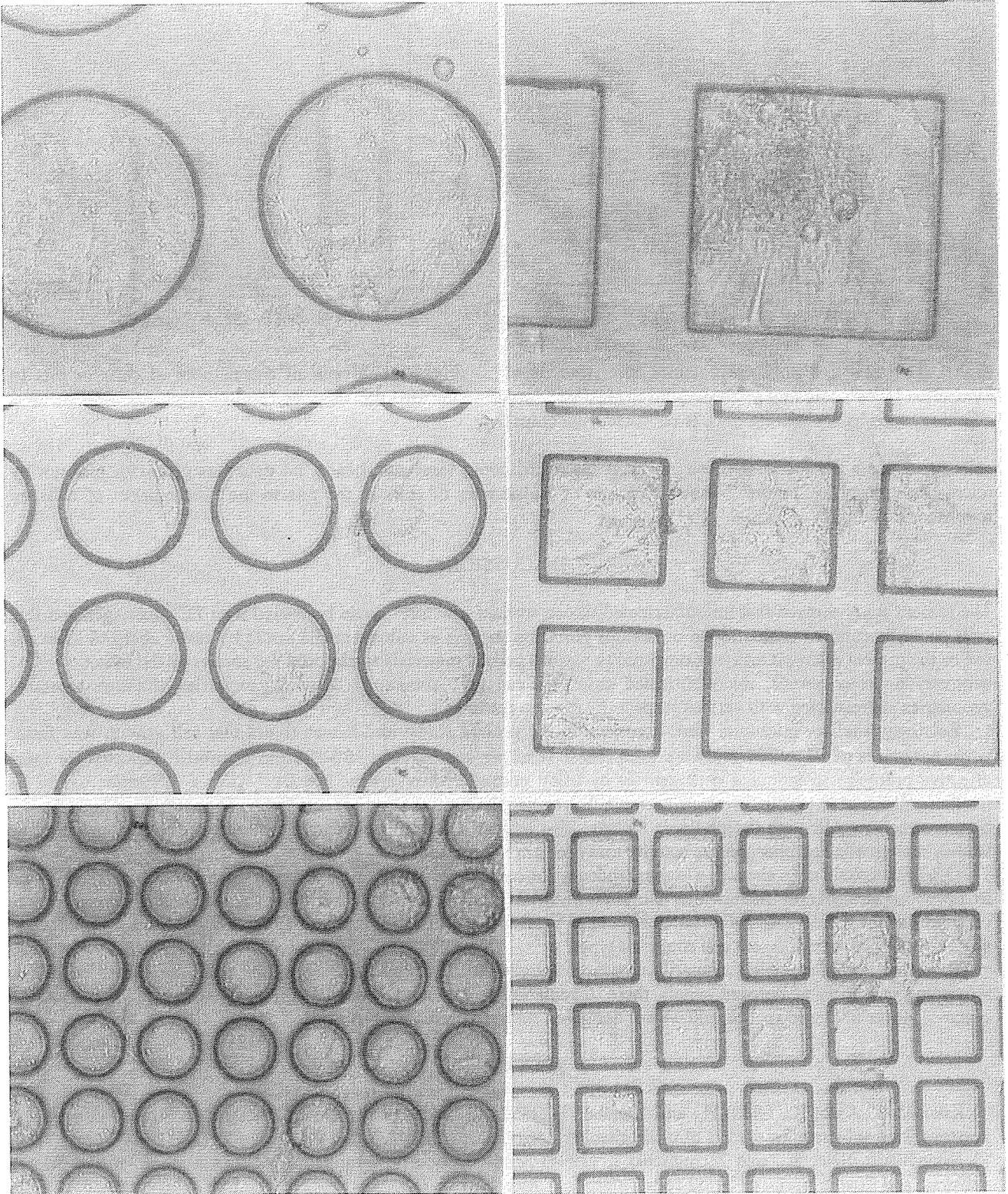


Fig. 5. Bone marrow derived cells incubated for 9 days in different size micropit arrays (upper, 200 μm ; middle, 100 μm ; lower, 50 μm).

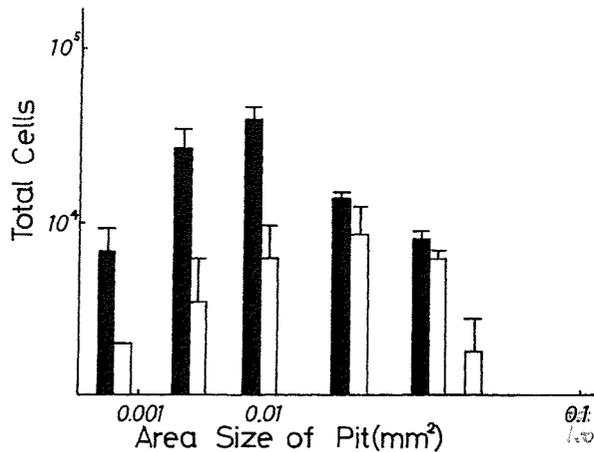


Fig. 6. The total cell numbers obtained after four days incubation by two counting methods (closed bars, microscopic counting; open bars, counting of cells detached from chips).

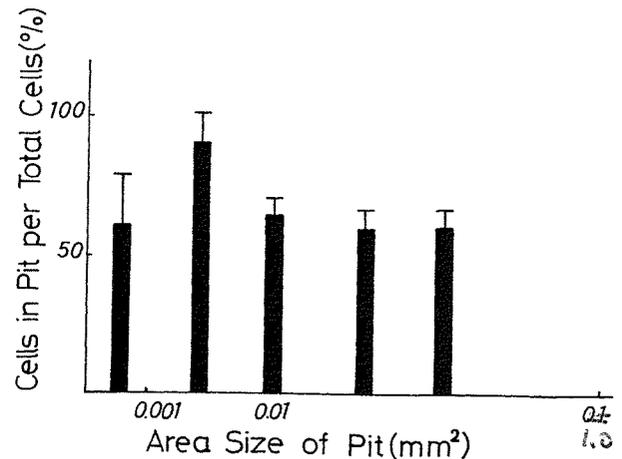


Fig. 7. The ratio of the number of cells in pits to the total cell number.

cells for 600 μm pits because of no marks in the microscopic view and, hence, no data was given for 600 μm by microscopic counting. Figure 7 shows the ratio of the number of cells in the pits to the total number of cells by microscopic counting.

DISCUSSION

The present study showed that microfabricated silicon surface structures could be coated with ECM collagen and that cells could be cultured in them with no serious problems so long as culture media could be easily changed as in the use of the present micropit arrays. Compared to various porous materials so far used for studies of the effect of ECM geometry on osteogenesis, microfabricated structures can give accurately defined geometries although creatable geometries are restricted with various respects by process features.

Relatively shallow micropits were examined in the present study, and it was found that cell growth was faster when pore sizes of 50 to 200 μm were used. These sizes are smaller than 300-400 μm previously reported for most effective pore size in inducing osteogenesis by using porous hydroxyapatite blocks.¹⁰ Such a difference would be naturally attributable to the difference between the regular shallow pit structure and irregular or polymorphous deeper porous structure. Although morphologic changes also indicated faster osteogenesis for cells in the pits, quantitative studies such as alkaline phosphatase activity assay remain to be made.

Osteoblast strain cells showed a higher tendency for cells to voluntarily pass through the microchannels compared to fibroblast strain cells. Meanwhile, the latter showed a higher tendency for cells to stretch in the terrace area compared to the former. Microfabricated structures would be useful for further clarifying such possible differences in cell behavior between different cell strains or types.

Extended fibroblast cells voluntarily detached themselves from the terrace area, and this appeared to be due to worsening condition of nutrient supply there. Circulation of culture medium is obviously required for cells to be cultured longer in closed spaces, and we are designing such structures for further studies.

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