Response of Human Chondrocytes on Polymer Surfaces with Different Micropore Sizes for Tissue-Engineered Cartilage

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ABSTRACT: Response of chondrocytes on polymer surfaces is important for applications of tissue-engineered cartilage, and tissue engineering contains the interaction of cells on material surfaces. We examined the behavior of human chondrocytes cultured on polycarbonate (PC) membranes with different micropore sizes (0.2–8.0 μm in diameter). The adhesion and proliferation of chondrocytes were evaluated by measuring the number of attached cells after 1, 2, and 4 days of culture and morphological observations. It seems that the cell adhesion and proliferation were progressively inhibited, as the PC membranes had micropores with increasing size, probably due to surface discontinuities produced by track-etched pores. On the PC membrane with smaller micropore sizes, the cells seemed to override these surface discontinuities. Phenotype of chondrocyte was assessed by Safranin-O staining for anionic sulfated proteoglycans. Chondrocytes on the 8.0 μm-diameter membrane surface proliferated, on 8 days, formed morphology of round shape, and expressed proteoglycans, because of limitation of spreading by the track-etched micropores. They maintained their phenotype under conditions that support a round cell shape on the large pore diameter substrate, whereas chondrocyte phenotypes lost on the small pore diameter membranes. In conclusion, this study demonstrated that micropore sizes as well as pore-to-pore distance play an important role for adhesion, proliferation, and phenotype of human articular chondrocytes. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 92: 2784–2790, 2004

Key words: adhesion; chondrocyte; micropore size; interaction

INTRODUCTION

The host–implant material interactions are initiated by the physical and chemical reaction that occurs as the material surface is conditioned by body fluid. Therefore, the interaction of cells with the polymer surface used in implant devices is important for a variety of biomedical and biotechnology application. The term of tissue engineering is coined to represent the construction in the laboratory of a device containing selective viable cells, biological signaling molecules, and scaffolds in a synthetic or biologic matrix that can be implanted in patients to promote regeneration of damaged tissue.[1–6] *In vitro* and *in vivo* tissue engineering involves the interaction of cells on material surfaces, because surface properties of materials will influence the initial cellular contact at the cell–material interface.

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In many cases, cells are sensitive to surface topography, and a wide variety of cell properties can be affected. Surface topography may affect the formation of a fibrous capsule around implants, inflammatory response at an implant–tissue interface, fibroblast attachment, angiogenesis, epithelial downgrowth around percutaneous devices, and many cellular processes such as cellular differentiation, DNA/RNA transcription, cell metabolism, and protein production.[7,8] The surface topography, which can influence the cellular interactions includes roughness,[9–14] micropore,[15] or microgroove,[16,17] and curvature.[18] Surface topography appears to be an important but often neglected factor in implant performance.

Surface properties of the biodegradable polymer used in tissue engineering on the interaction of cells are one of the most important parameters when biomaterials of implant devices for tissue engineering are designed. It is recognized that the behavior of the adhesion and proliferation of different types of cells on polymer materials depend on the surface characteristics such as wettability, chemistry, charge, roughness, and rigidity.[15,19–33]

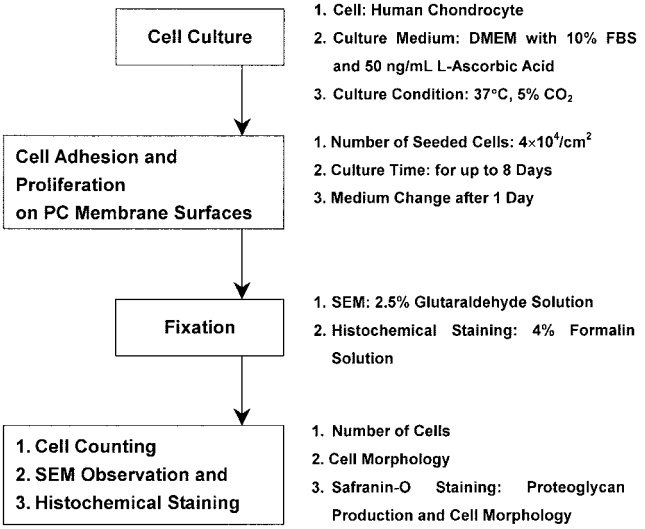

Figure 1 Experimental scheme for cell adhesion and proliferation test.

To demonstrate how tissue-engineered material surfaces influence cells, this article will examine studies of chondrocytes on polymer surfaces with different micropore sizes of the membrane. The purpose of the present study was to examine the effect of micropore size as well as pore-to-pore distance on changes of cell phenotypes and metabolism. Human chondrocytes were cultured on a range of porous polycarbonate (PC) membranes with well-defined surface topography as an attempt to investigate the effect of surface topography on the interaction of cells to polymeric materials.

EXPERIMENTAL

Materials

Commercially available PC membrane sheets (Poretics, USA) were used. These PC membranes have smooth and flat surfaces with track-etched uniform cylindrical pores. The membranes with a range of micropore sizes (0.2, 0.4, 1.0, 3.0, 5.0, and 8.0 μm in diamater) were selected for this study. The PC membranes were ultrasonically cleaned twice in ethanol for 30 min each, and then dried at room temperature in a clean bench. They were stored in a vacuum oven until use. The surface morphology of the PC membranes was examined by a scanning electron microscope (SEM; Model 2250N, Hitachi, Japan) after gold deposition in vacuum state.

Isolation and culture of human chondrocytes

Human cartilage was harvested from the articular joints of adult volunteers (age 18–38, male). Human chondrocytes were isolated from the tissue with enzymatic digestion. The cartilage was dissected and minced into 1–2 mm^3 pieces. After being rinsed twice in phosphate-buffered saline (PBS; Gibco Laboratories, Rockville, MD), the cartilage slices were digested with 0.3% type II collagenase (Sigma Chem. Co., St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories) supplemented with antibiotic-antimycotic (10,000 units penicillin G sodium, 10,000 μg streptomycin sulfate, and 25 μg amphotercin B in saline; Gibco Laboratories) under shaking for 4–6 h at 37°C. The digestion solution was filtered through a sterile cell strainer (100 μm nylon; Falcon, Lincoln Park, NJ) to remove any undigested fragments, and the human chondrocytes were subsequently collected by centrifugation (at 1500 rpm for 10 min) and washed twice with PBS. The human chondrocytes were cultured in tissue culture polystyrene (PS) flasks (Falcon) at 37°C under 5% CO_2 atmosphere in DMEM with L-glutamine, supplemented 10% fetal bovine serum (FBS; Gibco Laboratories), antibiotic-antimycotic, and 50 ng/mL L-ascorbic acid (Sigma Chem. Co.), and were harvested after the treatment with 0.05% trypsin-EDTA (Gibco Laboratories).

Chondrocyte adhesion and proliferation

The human chondrocytes were used to study the effects of surface topography as different micropore sizes on the adhesion and proliferation behavior of cultured cells. The cells were harvested after treatment with 0.25% trypsin-EDTA. The PC membranes were mounted in test chambers were equilibrated with prewarmed (37°C) PBS free of Ca^{2+} and Mg^{2+} for 30 min. The pores of the PC membranes were filled with the PBS during this step. After removing the PBS solution from the chambers by pippetting, the human chondrocytes (4×10^4 cells/cm^2) were seeded onto the PC membrane surfaces. The culture medium used was DMEM with L-glutamine, supplemented 10% FBS, antibiotic-antimycotic, and 50 ng/mL L-ascorbic acid. The cell culture on the PC membrane surfaces was carried out for up to 8 days. The culture medium was changed after 1 day.

SEM observation

To observe cell morphologies, after incubation at 37°C under 5% CO_2 atmosphere, the surfaces were washed with PBS and the cells attached on the surfaces were fixed with 2.5% glutaraldehyde (Sigma Chem. Co.) in PBS for 24 h at room temperature. After thorough washing with PBS, the cells on the surfaces were dehydrated in ethanol graded series (50, 60, 70, 80, 90, and 100%) for 10 min each and allowed to dry in a clean bench at room temperature. The cell-attached surfaces were gold deposited with plasma sputter (Model SC 500K, Emscope, UK) and examined by SEM with a tilt angle of 45 degree.

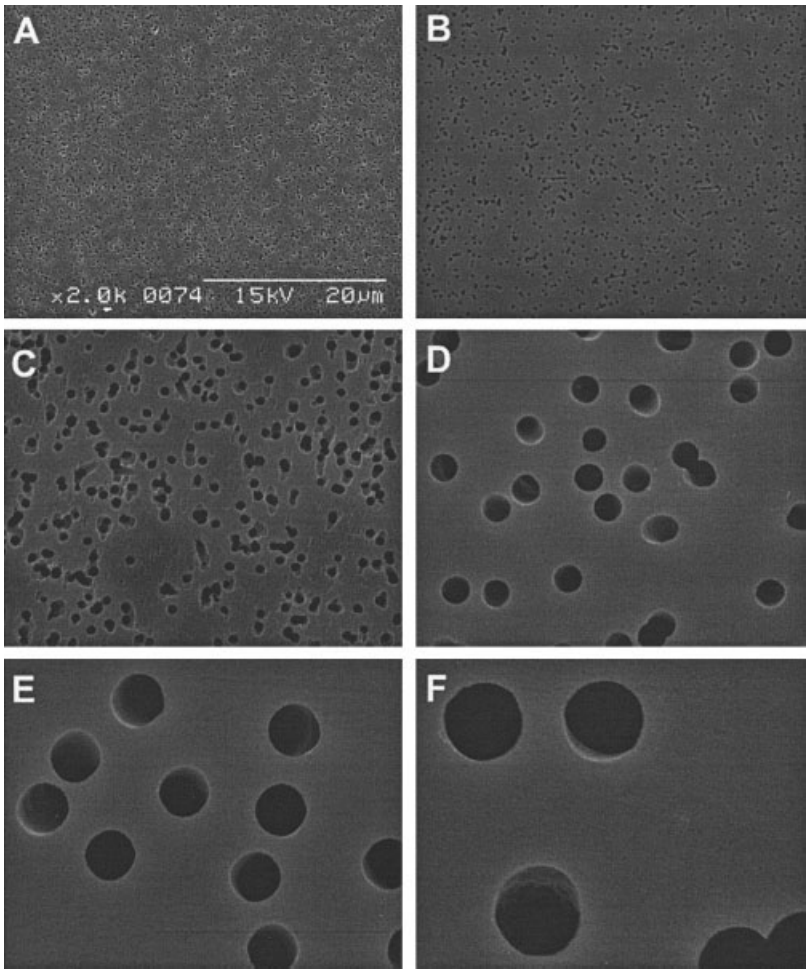

Figure 2 SEM pictures of PC membrane surfaces with different micropore sizes: (A) 0.2, (B) 0.4, (C) 1.0, (D) 3.0, (E) 5.0, and (F) 8.0 μm micropores (original magnification; $\times$2000).

Counting of cell number

Cultured cells onto PC membranes were counted 1, 2, and 4 days after seeding. All samples were washed with PBS, and cultured cells were retrieved by enzymatic dissociation with 0.25% trypsin-EDTA for 5 min for 37°C, followed by the addition of DMEM containing 10% FBS to stop the reaction. Cells were separated from the digest by centrifugation at 50 $\times$ g for 3 min. Cell pellets were washed with PBS, resuspended in PBS, and counted in a hemocytometer (Hausser Scientific, Livingston, NJ). The experimental scheme for cell adhesion and proliferation on the PC membrane surfaces with different pore size was summarized in Figure 1.

Histocytochemical staining

The cell-attached surfaces were fixed 4% formalin solution (Sigma Chem. Co.) for overnight and then fixed

TABLE I
Dimension of PC membranes[15]

Pore diameter (μm)	No. of pores (per mm^2)	Ave. pore-to-pore distance (μm)	Total pore area (mm^2/mm^2)	% of area available for cell attachment[a]
0.2	3.50×10^6	0.49	0.108	89.2
0.4	8.25×10^5	0.64	0.103	89.7
1.0	1.78×10^5	1.94	0.139	86.1
3.0	1.88×10^4	5.67	0.132	86.8
5.0	3.70×10^3	13.89	0.072	92.8
8.0	1.23×10^3	23.61	0.062	93.8

[a] % of area available for cell attachment = (total membrane area − total pore area)/(total membrane area).

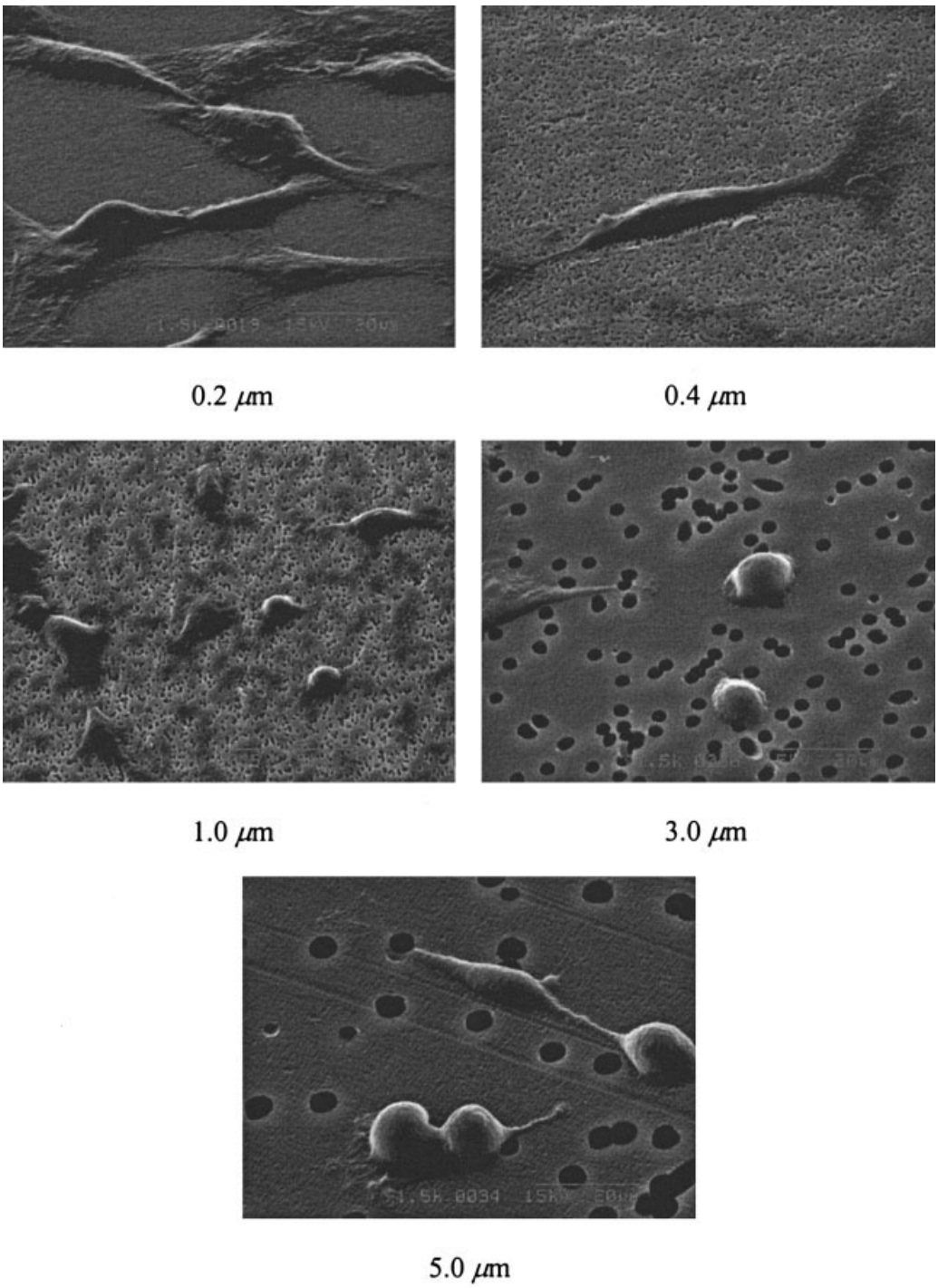

Figure 3 SEM pictures of human chondrocytes attached on PC membrane surfaces with different micropore sizes.

samples were transferred into 70% ethanol, dehydrated in ethanol and xylene series. Anionic sulfated proteoglycans of chondrocytes were detected by Safranin-O staining. Photomicrographs were taken using a Nikon inverted microscope with 40 and 100 magnifications.

Statistical analysis

All data are presented as mean ± SD of quadruplicate cultures. Statistic analysis was performed by Student's *t*-test (independent-difference). Results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of the PC membrane surfaces with different pore sizes

The PC membrane surfaces with different pore sizes such as 0.2, 0.4, 1.0, 3.0, 5.0, and 8.0 μm in diameter

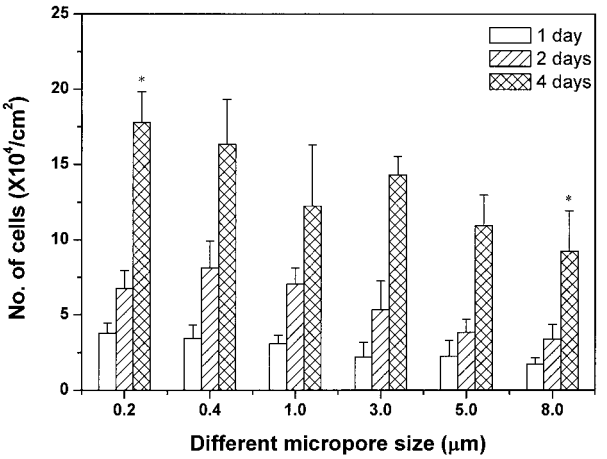

Figure 4 Human chondrocyte adhesion and proliferation on PC membrane surfaces with different micropore sizes (*$p < 0.05$, $n = 3$).

observed by SEM were smooth, and had randomly distributed circular pores, as seen in Figure 2. The dimensions of the PC membranes with different micropore sizes are given in Table I.[15] It shows that with an increase in membrane pore diameter, the number of pores per mm^2 (i.e., pore density) increased and the pore-to-pore distance decreased. The total pore area was in a range of 0.062 to 0.139 mm^2 per membrane surface area mm^2. The percent of area available for cell attachment was not significantly different regardless of pore sizes (86.1 to 93.8 %).

Chondrocyte adhesion, proliferation, and phenotype

To observe the effect of pore size and pore-to-pore distance of substrate on the adhesion and proliferation behavior of cells, human chondrocytes were cultured on the PC membrane surfaces with different micropore sizes for 1, 2, and 4 days. The culture medium was changed after 1 day. After 2 days of culture, the cell morphologies differed along the micropore size of membrane. The cells were protruded fillodopia and lamellapodia resulting in being less spread and flattened on large micropore surfaces than small ones. It seems that the cell adhesion and proliferation were progressively inhibited as the PC membrane surfaces had micropores with increasing size, probably due to surface discontinuities produced by track-etched micropores. On the PC membrane with 0.2, 0.4, and 1.0 μm-micropore sizes, the chondrocytes seemed to override these surface discontinuities, as seen in Figure 3.

The effect of micropore size on cell adhesion (after 1 day) and proliferation (after 2 days) behavior is shown in Figure 4. It was observed that the cell adhesion and proliferation decreased gradually with increasing micropore size of the PC membrane surfaces, even though the percentage of area available for cell attachment was not significantly different regardless of micropore size (86.1–93.8%, see Table I). In a previous study,[9] fibroblasts were cultured on a range of porous PC membranes. It was observed that cell adhesion and growth decreased gradually with increasing micropore size of the PC membrane surfaces. It seems that the cell adhesion and proliferation were progressively inhibited as the membrane surfaces had micropores with increasing size, probably due to surface discontinuities produced by track-etched pores. On the PC membrane surface with smaller micropore sizes, the cells seemed to override these surface discontinuities.

Ordinarily, chondrocytes lost their phenotype when plated on substratum, allowing cell spreading. Conversely, they maintained their phenotype under conditions that support a round cell shape.[12] Therefore, cell morphologies have something to do with chondrocyte phenotype. Proteoglycan production and morphologies of human chondrocytes on the PC membrane surfaces with 0.2 and 8.0 μm are shown in Figure 5. Particularly, human chondrocytes in 8.0 μm-micropore size PC membrane accumulated large amounts of intensely stained ECM that stained positively for anionic proteoglycans and maintained round-shape morphology after 8 days.

Figure 6 shows the schematic illustration depicting the mechanisms of cell interaction on the surfaces with different micropore sizes. On the small micorpore PC membrane with 0.2–1.0 μm in diameter (the same as 0.49 μm of average pore-to-pore distance), the cells were easily adhered and spread membrane surface like a fibroblast cell morphology. However, on the large micropore membrane with 3.0–8.0 μm in diameter (the same as 23.61 μm of average pore-to-pore distance), the cells appeared round-shape morphologies with phenotype of articular cartilage.

In summary, it can be observed that adhesion and proliferation of human chondrocytes prefer smaller pore surfaces to larger pore ones, but the maintenance of phenotypic morphology of human chondrocytes appears more in larger pore surfaces than small ones. In the case of the culture of rabbit chondrocytes on the polystyrene culture dish as a totally smooth surface, typical phenotypic morphology of these cells were observed first and second passages culture, whereas fibroblastic morphology of chondrocyte cells were observed after the third to fifth passages, that is to say, they lost their own phenotype along culture passages. Hence, it might be concluded that the larger pore surfaces tend to retain their own phenotypic morphology the same with totally smooth surface.[34]

Similar studies also have been investigated by other research groups[35,36] even though it was reported that osteoblasts or macrophages prefer roughened surfaces

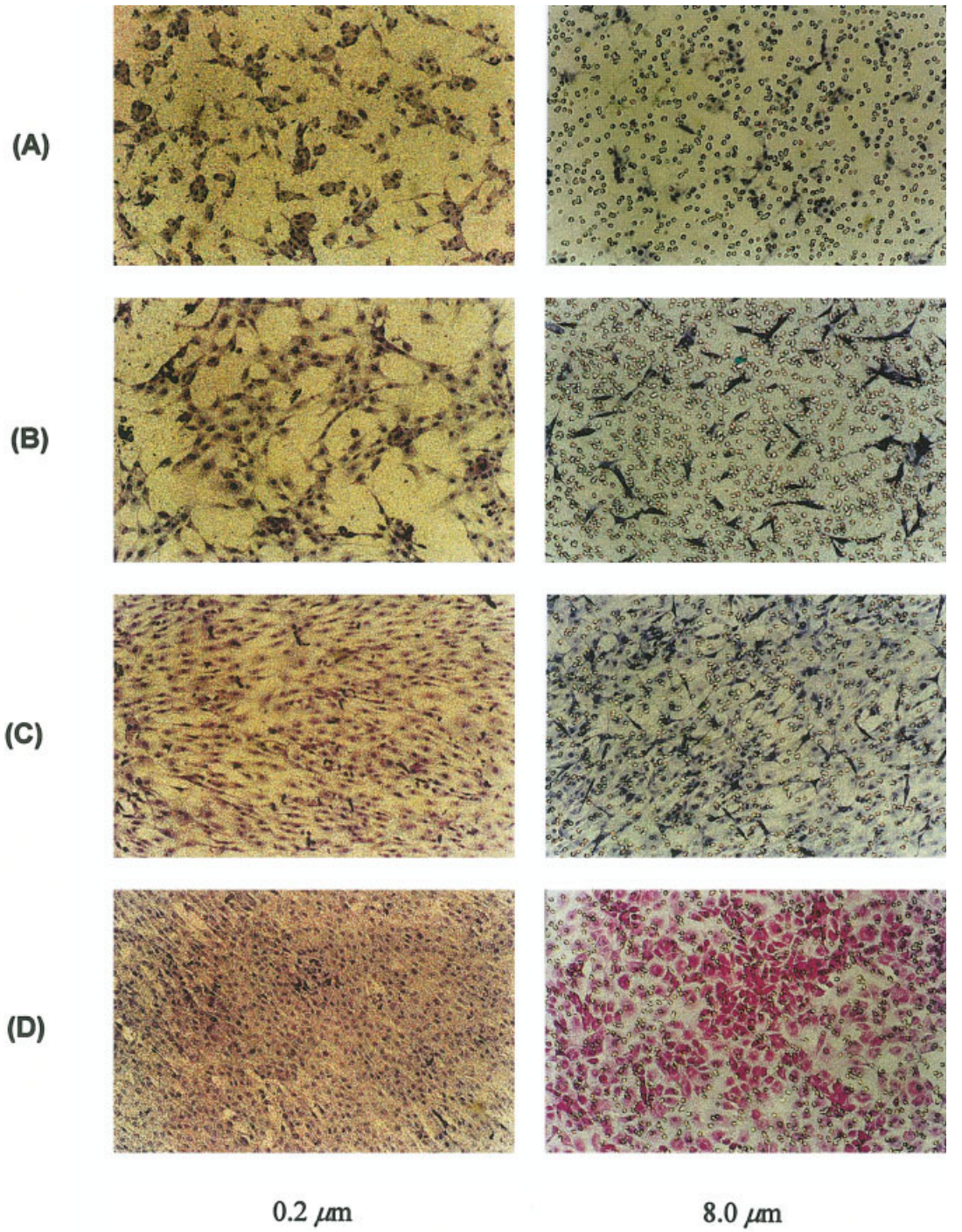

Figure 5 Histological evaluation of human chondrocyte phenotype by safranin-O staining; (A) 1, (B) 2, (C) 4, and (D) 8 days culture (×40, $n = 3$).

to smooth ones,[36,37] that oral epithelial cells are adhered slightly more on roughened surfaces than smooth ones, although the effect is not significant.[38,39] It seems that surface topography can affect cell adhesion and proliferation, but the particular effect can depend on cell types. The fact that different cell types react differently to surface topography is of extreme importance in implant design because more than one cell type is expected to interact with the surface. However, cell–surface interaction is a very complicated phenomenon, and thus it is still not clear why different cell types react differently to surface topography.

Studies on biochemical analysis as immunohistochemical staining for phenotypic expression, RT-PCR

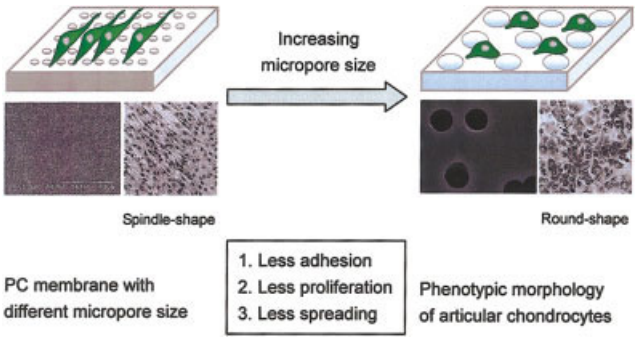

Figure 6 Schematic illustrations depicting the effect of the micropore size of the membrane on the cell adhesion and proliferation behavior.

for collagen type II and aggrecan, DNA quantification for cell proliferation, and so on, to assess the state of cells are in progress.

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