Proliferation Rate of Fibroblast Cells on Polyethylene Surfaces with Wettability Gradient

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ABSTRACT: Surface wettability on anchorage-dependent cells has an important role in cell growth rate. In our previous studies, we prepared a wettability gradient on polyethylene (PE) surfaces using corona discharge treatment from a knife-type electrode whose power increased gradually along the sample length. The PE surfaces were oxidized gradually with increasing power and characterized by Fourier transform infrared spectroscopy, contact angle goniometry, and electron spectroscopy for chemical analysis. The purpose of this study is to determine the rate of proliferation on polymer surfaces with different wettability. The behavior of cell growth for NIH/3T3 fibroblast cells attached on the polymer surfaces with different hydrophilicity was investigated using wettability gradient PE surfaces prepared by corona discharge treatment. They were investigated for the number of grown cells from 24 to 60 h in terms of surface wettability. From the slope of cell number on PE gradient surface versus culture time, the proliferation rates (number of cell/cm^2 · h) were calculated. It was observed that the proliferation rate was increased more on positions with moderate hydrophilicity of the wettability gradient surface than on the more hydrophobic or hydrophilic positions, i.e., 1111 (number of cell/cm^2 · h) of 57° of water contact angle at the 2.5-cm position ($P < 0.05$). This result seems closely related to the serum protein adsorption on the surface: the serum proteins were also adsorbed more on the moderately hydrophilic surface. In conclusion, surface wettability plays an important role in cell adhesion, spreading, and proliferation on the polymer surfaces. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 92: 599–606, 2004

Key words: wettability gradient; corona discharge; fibroblast cell; proliferation rate; protein adsorption

INTRODUCTION

Proliferation of anchorage-dependent mammalian cells is an important process for applications in many biochemical, biomedical, and tissue engineering areas.[1] Such applications include the reendothelialization of vascular prostheses, wound healing, and the growth of cells on biodegradable polymers for the regeneration of cartilage or other substrates in bioreactors as well as the production of proteins as monoclonal antibodies, interferon, and lymphokines.[2–7] Cell migration is an essential part of normal development, inflammation, tissue generation and repair, angiogenesis, and tumor invasion.[8]

The behavior of proliferation and migration of anchorage-dependent cells and tissue on polymeric materials depends on the surface characteristics of an extracellular matrix such as wettability (hydrophilicity/hydrophobicity or surface free energy), chemistry, charge, roughness, and rigidity.[9–16] Other important factors are the types of cell, the cell seeding density, the types of bioactive molecules such as cytokines in the cell culture medium, and the cell culture methods such as magnetic, optical, thermal, and electrical techniques.[17]

For investigation of the proliferation rate for different wettabilities, one problem, derived from the study using different kinds of polymers, is that the surfaces are heterogeneous both chemically and physically, which may result in considerable variation. Another methodological problem is that such studies are often tedious, laborious, and time consuming because a larger number of samples must be prepared to characterize the complete range of the desired surface property. The strong possibility of methodological error may also arise because the experiment for each sample is carried out separately.

In previous studies,[18–27] we proposed a wettability gradient on the polyethylene (PE) surfaces using a corona discharge treatment for the investigation of the

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interaction of the cell/polymer. The wettability gradient was produced by treating the PE sheet with corona from a knife-type electrode whose power was changed gradually along the sample length. The polymer surface oxidized gradually with increasing power and a wettability gradient was created on the PE surface. Also, this PE wettability gradient surface, and subsequent functional and chargeable chemogradient surfaces,[9,18–20] was used to investigate the interaction of different types of cells[26] [Chinese hamster ovary (CHO), fibroblast, endothelial cells, and pheochromocytoma[28] (PC-12)] and blood cells and proteins in terms of the surface hydrophilicity/hydrophobicity. We confirmed that the advantage of using wettability gradient surfaces as tools to study the interaction of biological species with solid surfaces is carrying the experiment at the same time and on the same surface. So, we tried to design the experimental apparatus for measurement of the proliferation rates of the fibroblast cells on a wettability PE gradient to function at the same time and on the same surface.

The aims of this study are (a) the preparation and characterization of a wettability gradient on a PE surface, (b) investigation of the effect of the proliferation rate of fibroblast cells on the wettability gradient PE surface for 24 to 60 h after cell seeding, and (c) investigation of the effect of the adsorption of serum protein on the proliferation rate of NIH/3T3 fibroblast cells, that is to say the growth rate of cells on PE surfaces was observed for the number of fibroblast cells in terms of surface wettability.

EXPERIMENTAL

Materials

An additive-free low-density PE sheet (50 to ~60 μm thickness, Hanyang Chemical Co., Korea) was used as the polymeric substrate for the preparation of wettability gradient surfaces. The transparent PE sheet was cut into 5 × 7 cm pieces, ultrasonically cleaned twice in ethanol for 30 min each, and then dried at room temperature on a clean bench. The pieces were stored in a vacuum oven until use. The cleanliness of the surface was verified by electron spectroscopy for chemical analysis (ESCA).

Preparation and characterization of wettability gradient surfaces

The PE film was treated with a radiofrequency (RF) corona discharge apparatus for the preparation of gradient surfaces in a manner similar to that used in our previous studies.[18–27] Briefly, a knife-type electrode was connected to the RF generator and the power was increased gradually by a motorized drive (Fig. 1). The cleaned PE film was placed on the sample bed and dry air was purged through the apparatus at a flow rate of 20 L/min. The electrode was 1.5 mm away from the sample bed and was translated at a constant speed, 1.0 cm/s; the corona from the electrode was discharged onto the sample with gradually increasing power (from 10 to 50 W at 100 kHz). The sample film (5.0 × 5.0 cm) was treated for 5 s. Using this treatment, the sample surface was continuously exposed to the corona with increasing power, resulting in the formation of a wettability gradient on the surface.

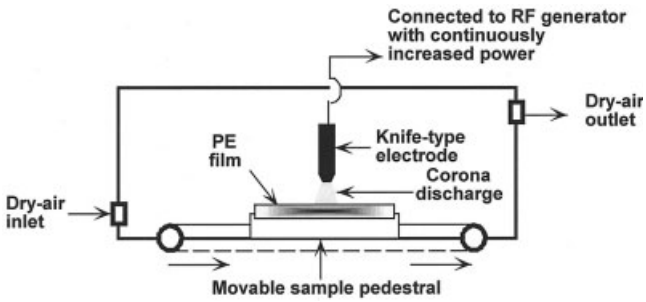

Figure 1 Schematic diagram showing the corona discharge apparatus for the preparation of wettability gradient surfaces.

The corona-treated PE surfaces were characterized by measurement of the water contact angle. The water contact angle, an indicator of the wettability of surfaces, was measured by a sessile drop method at room temperature using an optical bench-type contact angle goniometer (Model 100–0, Rame-Hart, Inc.). Drops of purified water, 3 μL, were deposited on the corona-treated PE surface along the sample length using a microsyringe attached to the goniometer. The amount of peroxides produced on the PE surface along the sample length by the corona discharge treatment was determined using the radical scavenger, 1,1-diphenyl-2-picrylhydrazyl (DPPH).[28,29] The corona-treated PE film was cut perpendicular to the gradient into five sections (1.0 cm each). Each section was immersed in 10 mL DPPH ethanol solution (1.0×10^{-4} mol/L) for 6 h at 70°C. The DPPH molecules consumed were quantified from the difference in transmittance at 520 nm between the untreated control and the corona-treated section. The adsorption coefficient of DPPH at 520 nm was 9.33×10^{-5}L/mol cm.

The changes in chemical structure of the gradient surface along the sample length were investigated by ESCA. The sheet was cut perpendicular to the gradient into five sections (1.0 cm each). The ESCA (ESCALAB MK II, V. G. Scientific Co., UK) was equipped with an Al $K\alpha$ radiation source at 1487 eV and 300 W power at the anode. Carbon 1S core level scan spectra were taken to analyze each section (analysis area, ~5 mm^2) of the gradient. The Fourier transform infrared with attenuated total reflectance (FTIR-ATR) spectra were obtained using a Magna-IR spectrometer 550 (Nicolet)

equipped with a KRS-5 internal reflection element (incidence angle, 75°). More details regarding the corona discharge apparatus and the preparation and characterization of wettability gradient surfaces were described in previous papers.[18–29]

Cell culture and measurement of proliferation rate on wettability PE gradient surfaces

NIH/3T3 fibroblast cells (KCLB 21,658) were obtained from Korean Cell Line Bank (Seoul, Korea) and used to study the effects of surface wettability of PE on the behavior of cultured cells. The cells, routinely cultured in tissue culture polystyrene flasks (Corning, Rochester, NY) at 37°C under 5% CO_2 atmosphere, were harvested after treatment with 0.25% trypsin (GIBCO BRL, Grand Island, NY). The PE gradient surface (size: 1.0 × 6.0 cm) was scratched on the back side of the PE sheet at 0.5, 1.5, 2.5, 3.5, and 4.5 cm with a middle line using the back of a knife to mark the position for taking photographs every 2 h and was then placed in the custom-made cell culture chamber as shown by the asterisk in Figure 2A. The PE gradient surfaces, placed on the culture plate with a silicon block, were equilibrated with Dulbecco's phosphate-buffered saline (PBS, pH 7.3~7.4; Sigma Chemical Co., Ltd., St. Louis, MO) free of Ca^{2+} and Mg^{2+} for 30 min. After the PBS solution was removed from the chambers by pipetting, the cells ($8 \times 10^4/cm^2$) were seeded to the surfaces. The culture medium used was RPMI 1640 nutrient mixture (GIBCO BRL) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL gentamycin sulfate.

The cell culture on the PE gradient surfaces was carried out for up to 60 h. The culture medium was changed once after 24 h. After incubation at 37°C under a 5% CO_2 atmosphere with a custom-made incubation chamber on the Nikon Diapot inverted microscope with 100× magnification and video tracking system shown in Figure 2B, the proliferation behavior on the surface was recorded by a VCR system at each section and then printed out every 2 h using a video print system. The grown fibroblast cell density on the surfaces was estimated by counting the number of attached cells. The same fields for each section were randomly counted every 2 h and the results were expressed in terms of the number of cells attached per square centimeter. The rate of proliferation, cell growth rate, was calculateed from the slope of the numbers of adhered cells versus culture time per square centimeter (No. cell/$cm^2 \cdot h$) from 24 to 60 h using the least-squares method.

Meanwhile, for taking the SEM picture for the cell morphology at each different wettability for 24 and 48 h, the surfaces were washed with PBS and the cells attached on the surfaces were fixed with 2.5% glutaraldehyde (GIBCO BRL) in PBS for 24 h at room temperature. After being thoroughly washed 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 on a clean bench at room temperature. The cell-attached PE gradient surfaces were gold deposited in a vacuum using plasma sputtering (SC 500K, Emscope, UK) and examined by a scanning electron microscope (SEM, Model *S*-2250*N*, Hitachi Co., Ltd., Japan) with a tilt angle of 45°. Further detailed procedures for the cell

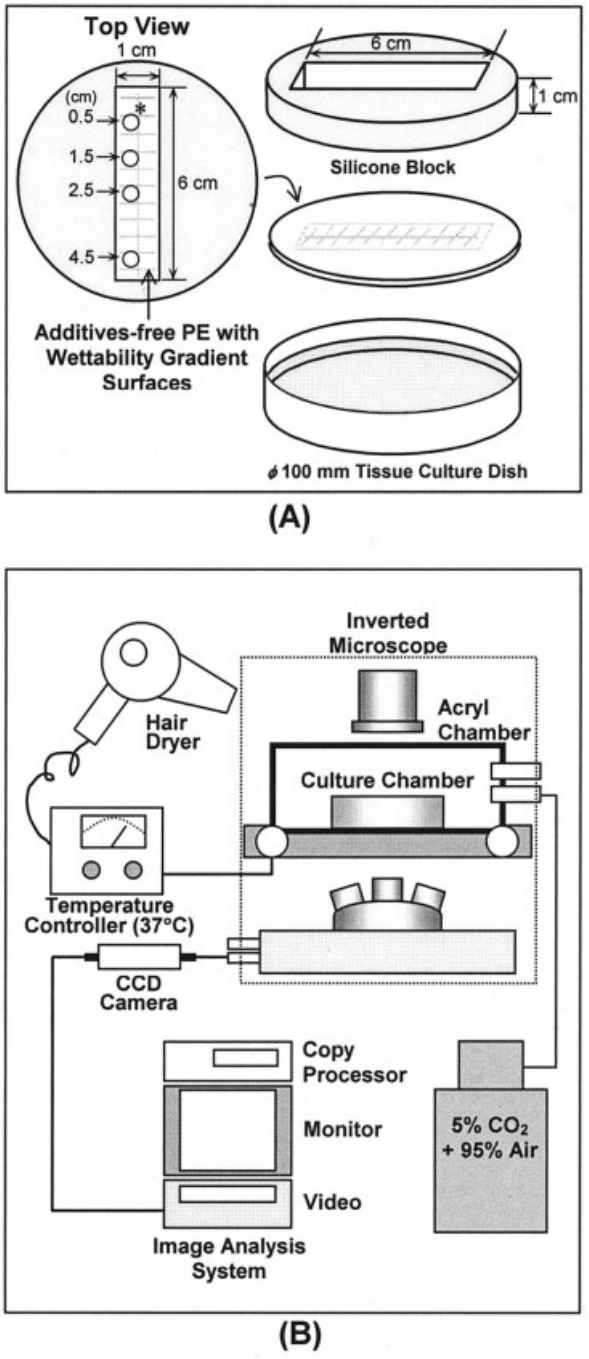

Figure 2 Schematic diagram of (A) a custom-made cell culture chamber for the wettability PE gradient surfaces in detail (* recorded area) and (B) the cell culture system on the inverted microscope and image analysis system.

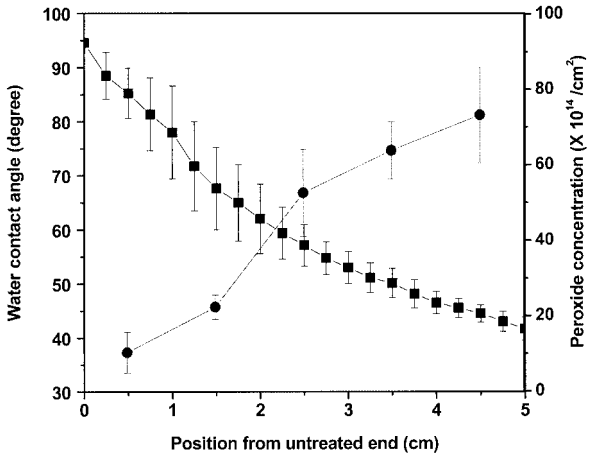

Figure 3 Changes in water contact angle (■) and peroxide concentration (●) of the corona-treated PE surface along the sample length. Sample numbers, $n = 3$.

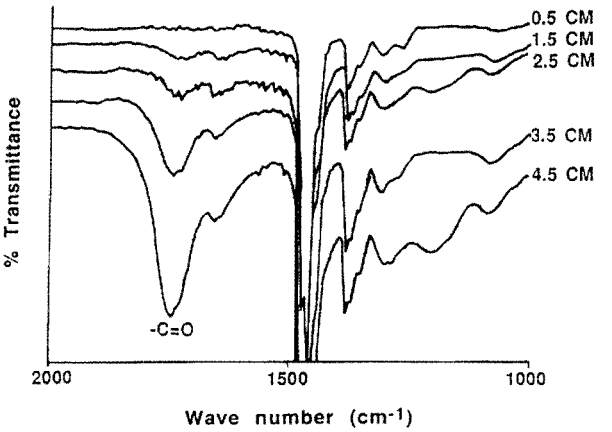

Figure 5 FTIR-ATR spectra of a corona-treated PE surface along the sample length. Numbers labeled on the spectra (0.5 to 4.5) represent the positions from the untreated end of the gradient surface.

culture on the PE gradient surfaces were described in previous papers.[18–29]

Serum protein adsorption

The same FBS added to the cell culture mediun was used to study the relationship between the behaviors of cell adhesion and serum protein adsorption on the PE gradient surfaces. FBS was diluted with PBS to make a 10% solution (same concentration as the cell culture medium). The PE gradient surfaces were mounted in the same test chambers used for cell adhesion tests. The surfaces mounted in the test chambers were equilibrated with PBS for 30 min. After the PBS solution was removed from the chambers, the FBS solution was introduced into the chambers. After 1 h of incubation at 37°C, the surfaces were washed with PBS and then washed with purified water to remove unadsorbed serum proteins. After being vacuum dried, the protein-adsorbed PE gradient surfaces were analyzed by ESCA. The nitrogen 1S peaks from the survey scan spectra were used for the analysis of proteins adsorbed on the PE gradient surface.[26]

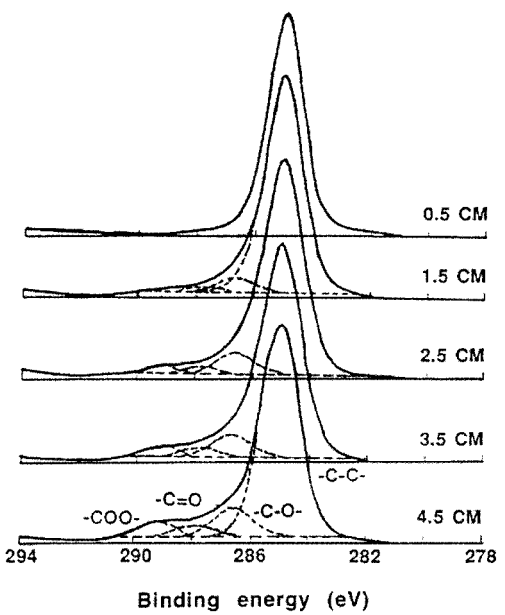

Figure 4 ESCA carbon 1S core level spectra of a corona-treated PE surface along the sample length. Numbers labeled on the spectra (0.5 to 4.5) represent the positions from the untreated end of the gradient surface.

Statistical analysis

Data are presented as means ± 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 wettability gradient surfaces

As Figure 3 indicates, the water contact angles of the PE surface gradually decreased along the sample

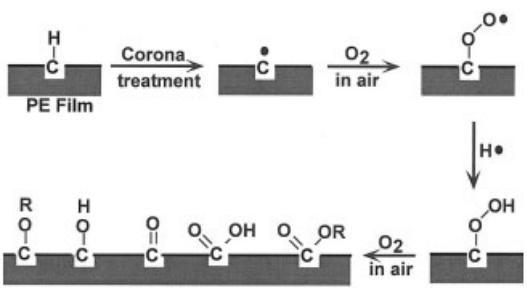

Figure 6 Possible mechanisms of the introduction of the oxygen-containing group after corona-treated PE.

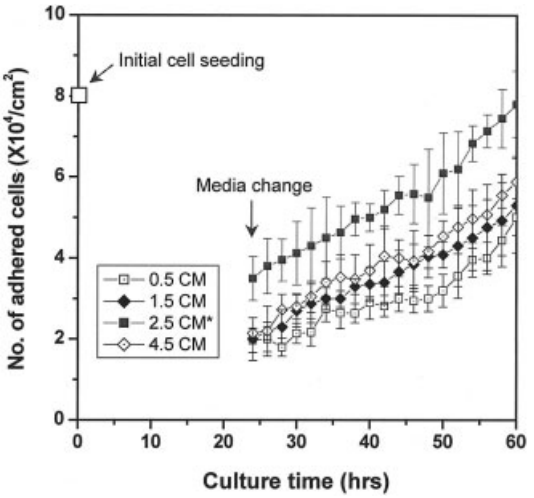

Figure 7 Growth behavior of fibroblast cells on wettability gradient PE surfaces from 24 and 60 h of culture (number of seeded cells, $8 \times 10^4/cm^2$). *$P < 0.05$.

TABLE I
Cell Growth Rate on the PE Wettability Gradient

Position on PE wettability gradient (cm)	Contact angle (°)	Cell growth rate (No. cell/cm^2 · h)
0.5	85	734
1.5	67	838
2.5[a]	57	1111
4.5	45	924

[a] $P < 0.05$ compared with another position.

length with increasing corona power from 96 to 43°. The amount of peroxide produced on the PE surface gradually increased along the sample length with increasing corona power. The decrease in the contact angles (and thus the increase in wettability) and the increase in the amount of peroxide concentration along the sample length may be due to oxygen-based polar functionalities incorporated on the surface by the corona discharge treatment. Figure 4 shows ESCA carbon 1S core level scan spectra of the corona-treated PE surface. The hydrophobic side of the gradient (position, 0.5 cm) showed an alkyl carbon (–*C*–C–) peak at a binding energy of ~285 eV. The positions with increasing corona power showed new peaks at higher binding energies, indicating the formation of a carbon–oxygen region corresponding to –*C*–O– at ~286.6 eV (e.g., hydroxyl or ether groups), —*C*=O at ~286 eV (e.g., ketone or aldehyde groups), and O=*C*–O— at ~289.1 eV (e.g., carboxylic acid or ester groups). The oxygen-based functional groups increased with increasing corona power. This was also confirmed by FTIR-ATR as shown in Figure 5. The peak heights of C=O at around 1700 cm^{-1} increased with increasing corona treatment power.

From contact angle, determination of hydroperoxide amount, ESCA, and FTIR-ATR, the possible mechanism of surface oxidation, which occurs on a polymer surface such as PE by corona discharge treatment, is depicted by a schematic diagram as shown in Figure 6. The corona discharge treatment of a polymer surface produces carbon radicals from the hydrocarbon backbone, followed by the formation of unstable hydroperoxides to produce various oxygen-based functional-

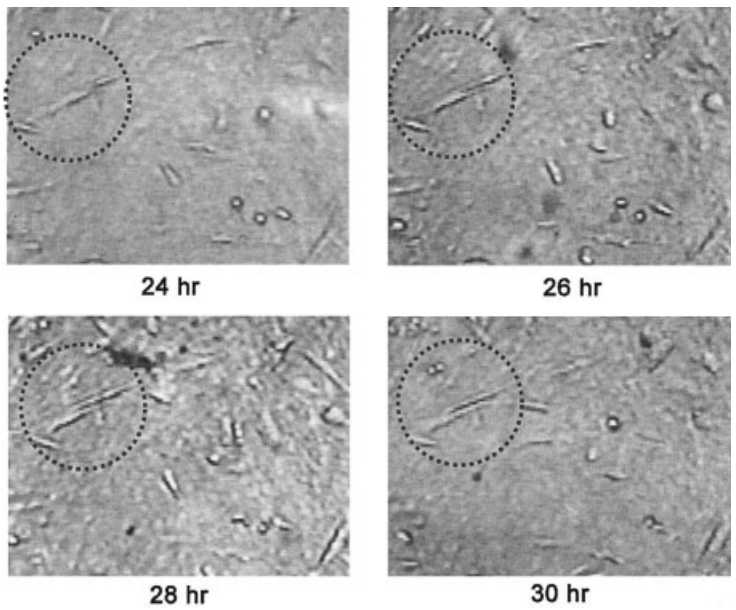

Figure 8 Typical recorded pictures through an inverted micrograph of the growing and migrated fibroblast cells at the 1.5-cm position for every 2 h on wettability gradient PE surfaces (original magnification, ×100).

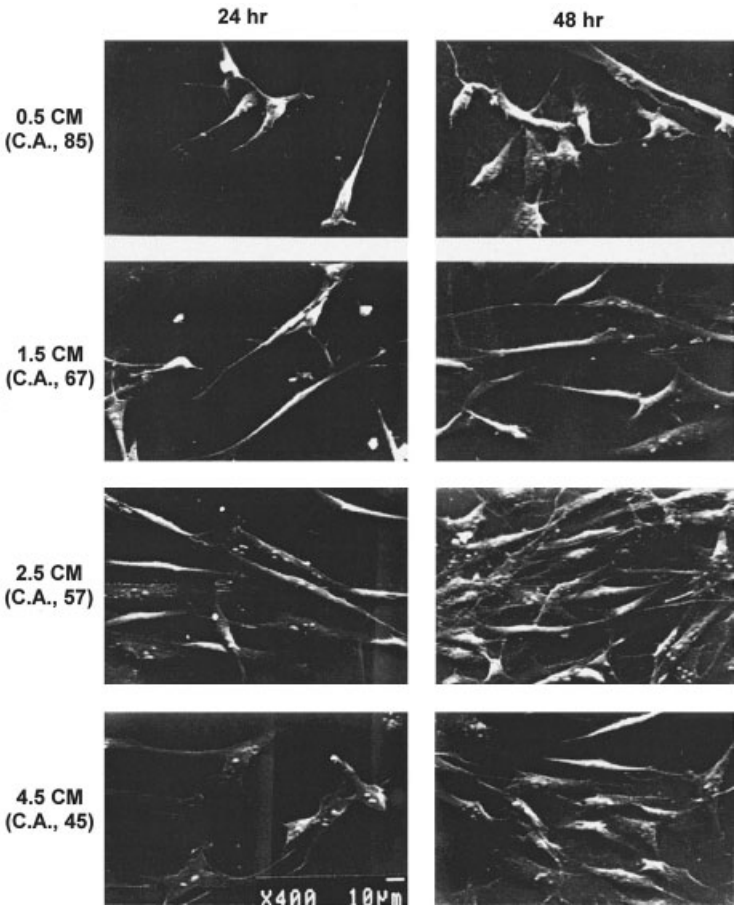

Figure 9 SEM pictures of the fibroblast cells adhered on wettability gradient PE surfaces after 24 and 48 h of culture (original magnification, ×400). CA, water contact angle (degrees).

ities (hydroxyl group, ether, ketone, aldehyde, carboxylic acid, carboxylic ester, and so on) by reaction with additional oxygen.[30–36]

Proliferation rate of fibroblast cells on wettability gradient surfaces

Figure 7 shows the growth behavior of fibroblast cells on the wettability gradient PE surface and Figure 8 shows a typical recorded picture of two migrated fibroblast cells at position 1.5 cm for 24 to 30 h after seeding. Good linearity was observed during the culture experiment ($r^2 = 0.99$) in Figure 7. The maximum number of growing cells appeared around position 2.5 cm (water contact angle, about 55°) compared with other regions as shown. That is to say, fibroblast cells grew significantly more on the positions with moderate hydrophilicity of the wettability gradient surface than on the more hydrophobic or hydrophilic positions. The proliferation rates of cell were calculated from each slope of the growth line using the least-squares method as listed in Table I. As shown in Table I, the no. cell/cm^2 · h of cell growth rate of 1111 was at maximum rate at position 2.5 cm ($P < 0.05$). More precise cell morphology, as observed by SEM, is shown in Figure 9. Fibroblast cells, with protruding fillodopia and lamelliopodia that spread out and flattened after 1 and 2 days of culture, were more on the 2.5-cm position with moderate hydrophilicity, whereas the control still showed relatively inactivated cell morphology. In our previous studies,[18–27] we observed that CHO, osteoblast (MG 63), hepatoma (Hep G2), bovine aortic endothelial (CPAE), and rat pheochromocytoma (PC-12) cells adhered and grew more in moderately hydrophilic positions of polyethylene, polycarbonate, poly(methylmethacrylate), polyurethane, and poly(L-lactide-*co*-glycolide) surfaces. Also, it was observed that endothelial cells adhered and grew more on positions with moderate hydrophilicity of the wettability gradient PE surface under the flow condition.[25,26] The endothelial cell adhesion strength was higher on the hydrophilic positions than on the hydrophobic ones. The maximum adhesion strength of the cells also appeared at water contact angles around 55°. Greater than 90% of the adhered cells remained in that position after applying a shear stress of 250 dyn/cm^2, for 2 h, whereas the cells were com-

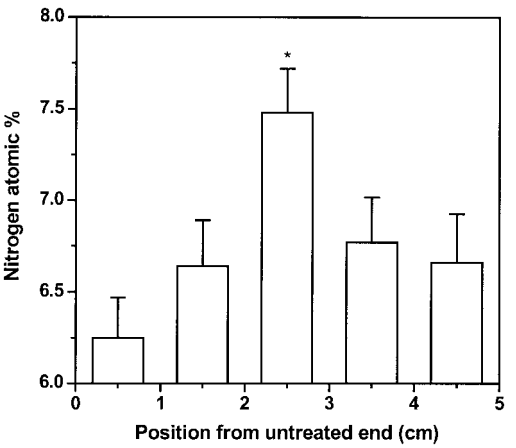

Figure 10 Serum protein adsorption on a wettability gradient PE surface. Nitrogen atomic % represents the relative amount of the proteins adsorbed on the surface (1 h adsorption in 10% FBS solution); $n = 3$, $^*P < 0.05$.

pletely detached on the hydrophobic position (water contact angle, about 86°) within 10 min after application of the same shear stress.

Serum protein adsorption

To see the effect of serum proteins in cell culture medium on the growth rate of fibroblast cells, FBS was adsorbed onto the wettability gradient PE surfaces for 1 h at 37°C. FBS was diluted with PBS to make a 10% solution, which is the same concentration as that in the cell culture medium. This method is a simple and easy method for obtaining semiquantitative information on protein adsorption. Since we investigated protein adsorption on polymer surfaces by ESCA and by using ^{125}I-labeled proteins,[16,26,28] we observed that protein adsorption on the surfaces, analyzed by both methods, showed almost the same trend. For ESCA analysis, the nitrogen peak (binding energy, ~399 eV) from the survey scan spectrum was used as an indicator of the protein adsorption on the surface, since it was observed that little nitrogen is incorporated onto the surface by corona treatment in air. It is mainly derived from peptide bonds of the adsorbed proteins. The ESCA spectra also showed an increased oxygen-to-carbon ratio after serum protein adsorption on the surface. Figure 10 shows the relative adsorbed amount of nitrogen atomic % of serum proteins on the wettability gradient surfaces. As the surface wettability increased along the sample length, the proteins adsorbed on the surface increased and then decreased; the proteins were adsorbed more on the positions with moderate hydrophilicity of the wettability gradient surface than more hydrophobic or hydrophilic ones. The maximum adsorption of the proteins appeared at around position 2.5 cm, which is the same trend seen for the proliferation rate of fibroblast cells. It can be explained that preferential adsorption of some serum proteins like fibronectin and vitronectin[9–15] from culture medium on the moderately wettable surfaces may be a reason for adhesion, spreading, and growth from fibroblast cells.

In conclusion, surface wettability plays an important role in the growth rate of anchorage-dependent cells on polymeric surfaces. We demonstrated in this study that the wettability gradient prepared on PE surfaces by the corona discharge treatment method can be a simple and effective tool for systematically investigating the role of surface hydrophilicity on the measurement of cell growth rate on sample surfaces. Also, the correlation of wettability, cell growth rate, and protein adsorption might be very useful for the design of biomaterials for applications such as the endothelialization of vascular graft and scaffold materials in the tissue engineering area.

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