Human monocyte adhesion and activation on crystalline polymers with different morphology and wettability *in vitro*

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Abstract: This study evaluated the effects of crystalline polyamide (Nylon-66), poly(ethylene-*co*-vinyl alcohol) (PEVA), and poly(vinylidene fluoride) (PVDF) polymers with nonporous and porous morphologies on the ability of monocytes to adhere and subsequently activate to produce IL-1β, IL-6, and tumor necrosis factor α. The results indicated monocyte adhesion and activation on a material might differ to a great extent, depending on the surface morphology and wettability. As the polymer wettability increases, the ability of monocytes to adhere increases but the ability to produce cytokines decreases. Similarly, these polymers, when prepared with porous surfaces, enhance monocyte adhesion but suppress monocyte release of cytokines. Therefore, the hydrophobic PVDF with a nonporous surface stimulates the most activity in adherent monocytes but shows the greatest inhibition of monocyte adhesion when compared with all of the other membranes. In contrast, the hydrophilic Nylon-66, which has a porous surface, is a relatively better substrate for this work. Therefore, monocyte behavior on a biomaterial may be influenced by a specific surface property. Based on this result, we propose that monocyte adhesion is regulated by a different mechanism than monocyte activation. Consequently, the generation of cytokines by monocytes is not proportional to the number of cells adherent to the surface. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 50, 490–498, 2000.

Key words: surface morphology; surface wettability; monocyte; adhesion; activation

INTRODUCTION

Recently, many polymeric materials have been applied clinically as biomaterials. Because implanted polymers are generally subjected to foreign body reactions by the host, the development of biocompatible polymers has been increasingly in demand. The interaction of inflammatory cells with biomaterials is believed to affect the biocompatibility of the biomaterials.[1–3] Among inflammatory cells, it is now accepted that monocytes play an important role in the foreign body reactions.[4,5] Cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF), that are released by activated monocytes can regulate fibroblast growth and induce other cells such as T lymphocytes to proliferate, synthesize proteins, and secrete factors, further activating macrophages that result in the so-called whole body inflammatory response.[6–10] Because cytokines contribute to and may augment the inflammatory response, it is of interest to evaluate the production of cytokines by monocytes in contact with biomaterials.

Some reports showed that the behavior of cells on a biomaterial is influenced by both their intrinsic genetic program and their extracellular environment.[11–14] Therefore, monocyte adhesion and activation on a biomaterial may present different characteristics and may be significantly influenced by the chemical nature and physical microstructure of the culture substrate. The combined effects of polymer substratum morphology and wettability on the monocyte adhesion and activation were examined in this study. Monocytes were cultured on poly(ethylene-*co*-vinyl alcohol) (PEVA), polyamide (Nylon-66), and poly(vinylidene fluoride) (PVDF) substrates because they had different wetta-

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bilities and different surface morphologies could be prepared. Monocyte adhesion was studied using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to evaluate cell viability. Monocyte activation was expressed as IL-1β, IL-6, and TNF-α secreted by MTT conversion. This work shows that the nonporous and hydrophobic surfaces attracted fewer adherent monocytes than porous and hydrophilic surfaces. However, the monocytes adhering to the nonporous and hydrophobic surfaces released higher amounts of cytokines than those adhering to the porous or hydrophilic surfaces. Based on this result, we hypothesize that interactions of monocytes with the biomaterial surface (adhesion and activation) are controlled by two different mechanisms.

MATERIALS AND METHODS

Membrane preparation and characterization

The polymer materials used in this study included PEVA (E105A containing ca. 56 mol % VA, Kuraray), Nylon-66 (Zytel 101, DuPont), and PVDF (Kynar 740, Elf Ato Chem). These polymer substrates were used in the membrane form with nonporous or porous surfaces. An appropriate amount of polymer was dissolved in a solvent to form a 25 wt % polymer solution. The solvents for PEVA, Nylon-66, and PVDF were DMSO, formic acid, and DMF, respectively. The polymer solution was spread on a glass plate in a uniform thickness of 100 μm using an autocoater (KCC303, RK Print-Coat Instruments) at 25°C to prepare membranes. Nonporous membranes were prepared using solvent evaporation in a vacuum oven at 80°C for 2 days. Porous membranes were prepared by immersing the casting solution into a 1-octanol bath for 2 days.[15] During this time of evaporation or precipitation, the casting solution became a solid membrane. Subsequently, the membrane was soaked in ethanol and water to remove any residual solvent.

The morphology of the membrane was examined using scanning electron microscopy (SEM). The freeze-dried samples were splattered with gold and palladium in a vacuum and scanned using a Hitachi S-800 microscope at 20 kV. The air–water contact angle of the membrane was measured at 25°C using a reverse air-bubble apparatus (CA-D, Kyowa Scientific Co.). Readings were made after the angles were observed to be stable with time. Six readings were made at different spots on each nonporous sample and averaged.

Monocyte culture

Venous blood was obtained from healthy adult volunteers in accordance with the guidelines of the National Taiwan University Hospital. Human peripheral blood mononuclear cells were isolated using the Percoll density gradient sedimentation procedure.[8,16] The mononuclear cells were harvested and washed once with cold phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco, Grand Island, NY) to minimize aggregation and resuspended in RPMI-1640 medium containing 10% fetal bovine serum (Sigma). The monocyte viability was > 85% as determined using trypan blue dye exclusion.

Monocyte cultures in the presence of the prepared membranes were established according to standard cell culture procedures. Circular samples (1.5-cm diameter) were cut from the membranes, rinsed extensively with distilled water, and sterilized under UV light overnight. Subsequently, circular samples were placed into the wells of 24-well tissue culture plates (TCPS, Corning, NY) by placing a sterilized Teflon ring on top of each sample to prevent the samples from floating. Then monocytes were added to the culture wells at a concentration of 1×10^5 cells/well in a volume of 1 mL/well RPMI, which contained 10% fetal bovine serum. In addition, lipopolysaccharide (LPS, 5 μg/mL) was added to mimic the foreign body reaction observed at the implanted biomaterial surfaces *in vivo*. Cells were also placed in empty polystyrene wells with a Teflon ring as reference wells. Reference wells were treated the same as test polymer containing wells. Cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

MTT assay

A colorimetric assay using MTT has been increasingly used to measure the viable cell number after the release of cytoplasmic contents into the medium from artificially lysed cells.[17] The MTT (Sigma) was prepared as a 0.5 mg/mL stock solution in PBS, sterilized using Millipore filtration, and kept in the dark. After 24 h of incubation, the wells were rinsed with PBS and MTT (0.35 mL) was added to each well and incubated for 5 h at 37°C. Mitochondrial dehydrogenases of viable cells cleaved selectively to the tetrazolium ring, yielding blue-purple formazan crystals. Therefore, the level of the reduction of MTT into a colored formazan salt in living cells was measured spectrophotometrically and reflected the number of viable cells present. After incubation the medium was aspirated and the formazan reaction products were dissolved in 0.4 mL of 10% sodium dodecyl sulfate (Sigma) in PBS and shaken for 15 h. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, Bio-Tek) at 570 nm. Viable cell numbers were determined using the MTT assay at 24 h after monocyte cultures. All experiments were repeated 4 times.

Cytokine assay

After 24 h of monocyte culture the supernatant was harvested and cytokines (IL-1β0 , IL-6, and TNF-α) released by monocytes into the medium were measured using the ELISA procedure following the manufacturer's directions

(Endogen, Inc., Boston). All experiments were repeated 4 times.

Cell morphology

Monocytes adhering to the membranes were washed with PBS after 24-h incubation. Subsequently, the cells were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C. They were then postfixed for 1 h in 1% osmium tetroxide at 25°C. After being thoroughly washed with PBS, the specimens were dehydrated using graded ethanol changes, critical point dried, gold splattered in a vacuum, and examined using SEM.

Data analysis

Data are presented as the mean ± standard deviation. Statistical significance was calculated using one way analysis of variance followed by Student's t test ($p < 0.05$ was considered significant).

RESULTS

Membrane morphology

Macroscopically, nonporous membranes were transparent and porous membranes were white and opaque. The microscopic analyses of membrane surface morphologies were evaluated using SEM. All nonporous membranes prepared by solvent evaporation had a similar morphological appearance of dense surfaces as indicated in Figure 1. Figure 2 shows the porous morphologies for membranes prepared by immersing the casting solution into a 1-octanol bath. It is interesting that, although PEVA, Nylon-66 and PVDF have rather different chemical properties, they all formed membranes with a particulate morphology as they were precipitated from 1-octanol.[18] The diameters of the particles were estimated to be approximately 0.3, 4, and 1μm for membranes PEVA, Nylon-66, and PVDF, respectively. Furthermore, these porous membranes consisted of larger pores among connected particles and smaller pores within each particle.[19] The small pores within the Nylon-66 particles were similar to the dendritic processes on the cell membrane of mature macrophages. It would be interesting to see whether this dendritic structure increases monocyte adhesion or reduces monocyte activation on the porous Nylon-66 membrane.

Contact angle analysis

The air–water contact angles were 22.1 ± 2.4°, 56.1 ± 1.8°, and 132.2 ± 2.4° for Nylon-66, PEVA, and PVDF, respectively. This shows that Nylon-66 and PVDF were the most hydrophilic and the most hydrophobic membranes, respectively. Because PEVA contains hydrophilic VA segments and hydrophobic ethylene segments, its wettability was intermediate between Nylon-66 and PVDF membranes.

Monocyte adhesion

Figure 3 shows the formazan accumulation for the membranes and TCPS at 24 h after plating. For nonporous membranes the PVDF significantly inhibited monocyte adhesion compared to TCPS ($p < 0.05$). The PEVA had a slight higher formazan absorbance than TCPS. Only Nylon-66 had a significant difference of accumulation of formazan compared with TCPS ($p < 0.05$). In contrast to nonporous membranes, the three porous membranes had higher accumulations of formazan and significant differences compared to TCPS ($p < 0.05$). This indicates that a membrane with porous morphology is favorable for the attachment of monocytes, regardless of the membrane water contact angle. In addition, as the membrane water contact angle decreases, the ability of the membranes to adhere to monocytes increases, regardless of the type of surface, nonporous or porous. In summary, the substrate morphology and wettability on which the cultured monocytes attach are both important factors affecting monocyte adhesion.

Cytokine production

Table I presents the amounts of IL-1β, IL-6, and TNF-α released by monocytes *in vitro,* including the exposures of prepared membranes with LPS into the medium after 24-h incubation. Membranes with different wettabilities and morphologies stimulated monocytes to produce different quantities of different cytokines. When the data were evaluated based on cytokine dependence, the quantities of cytokines produced followed the order IL-1β > IL-6 > TNF-α. Such a phenomenon was also reported by Bonfield et al. for cytokine production by monocytes on protein preadsorbed polymers.[20] However, contrary to our present result, Bonfield et al. pointed out that IL-6 was the cytokine produced by the monocytes in the greatest quantity.[20] In addition, when the data were evaluated based on membrane surface property dependence, it seems that surface wettability and morphology did

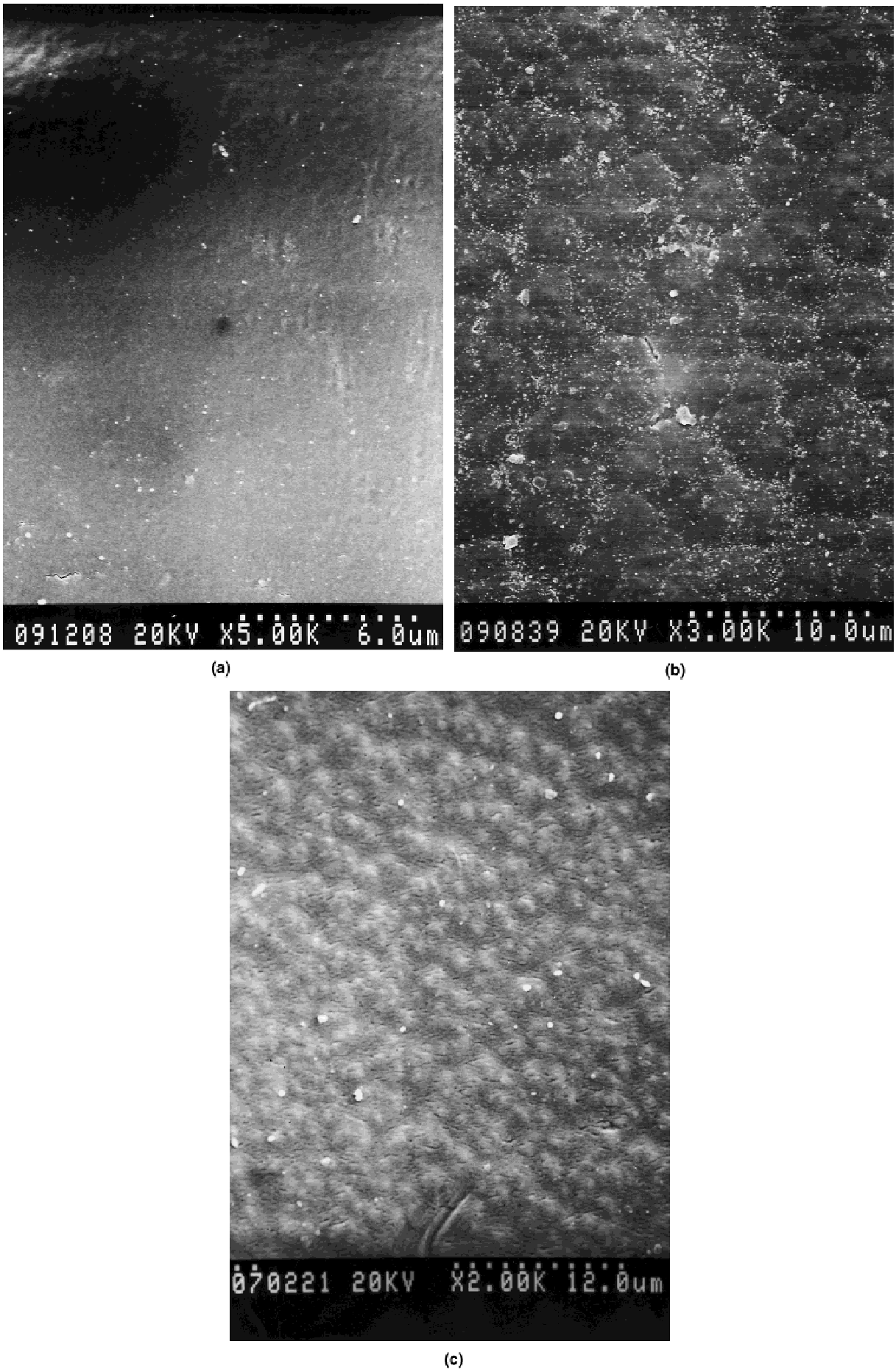


Figure 1. SEM photographs of nonporous surfaces of (a) PEVA, (b) Nylon-66, and (c) PVDF.

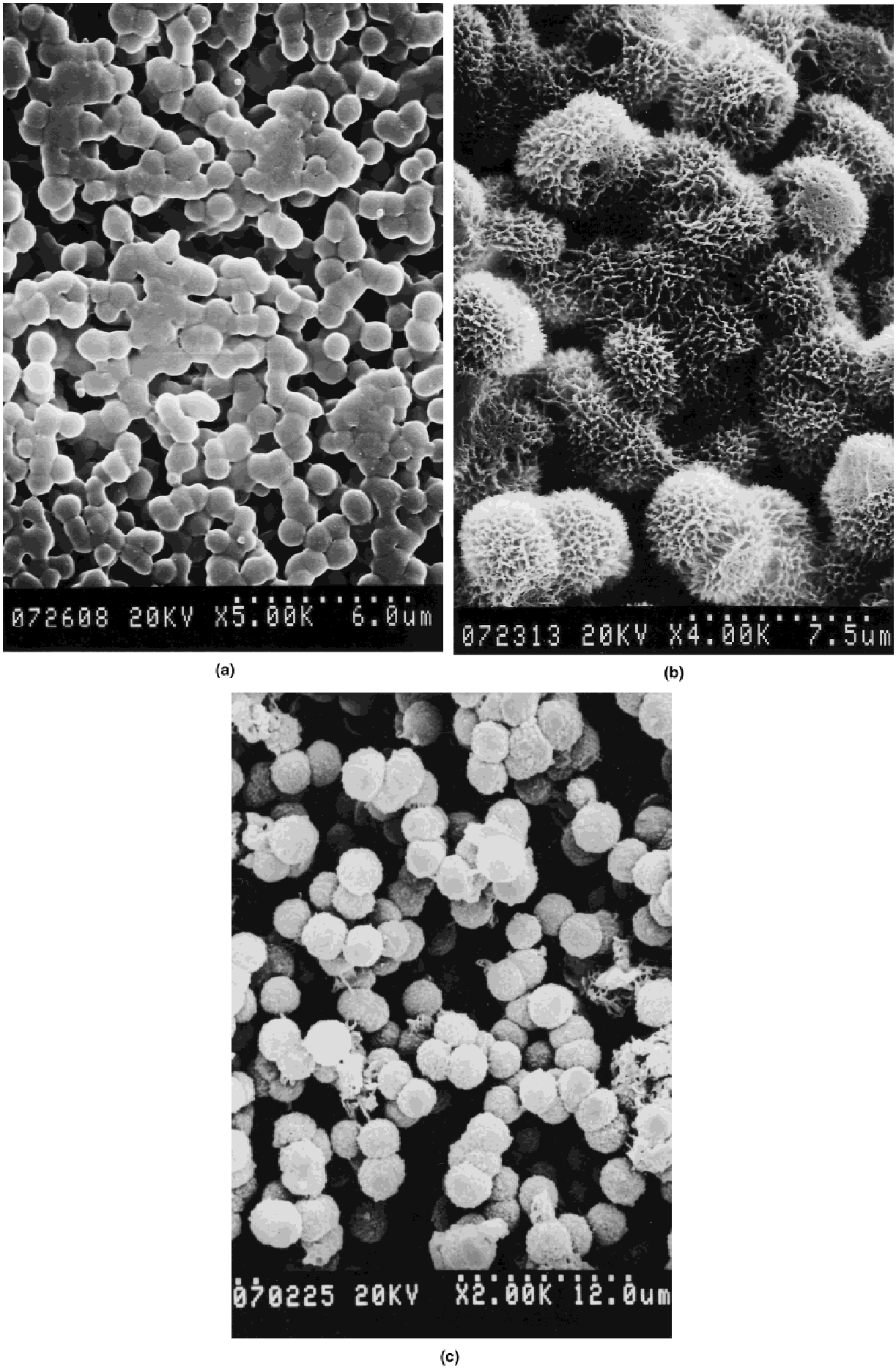


Figure 2. SEM photographs of porous surfaces of (a) PEVA, (b) Nylon-66, and (c) PVDF.

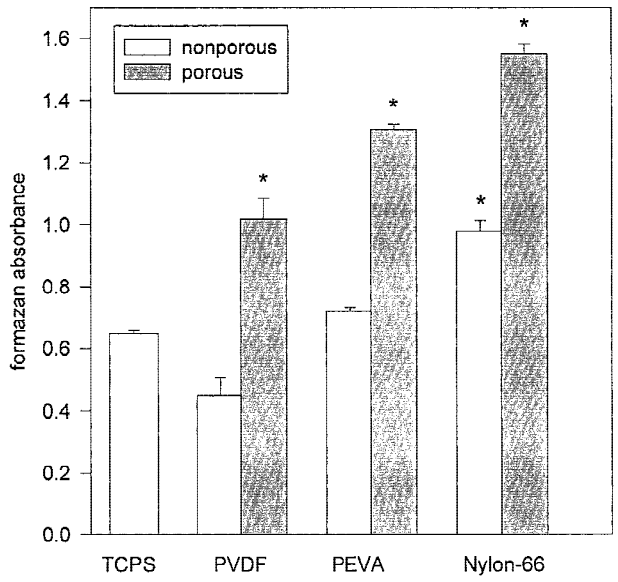


Figure 3. The formazan accumulation at 24 h from monocytes seeded onto membranes and TCPS ($n = 4$). *Significant differences ($p < 0.05$) of monocyte adhesion compared to TCPS.

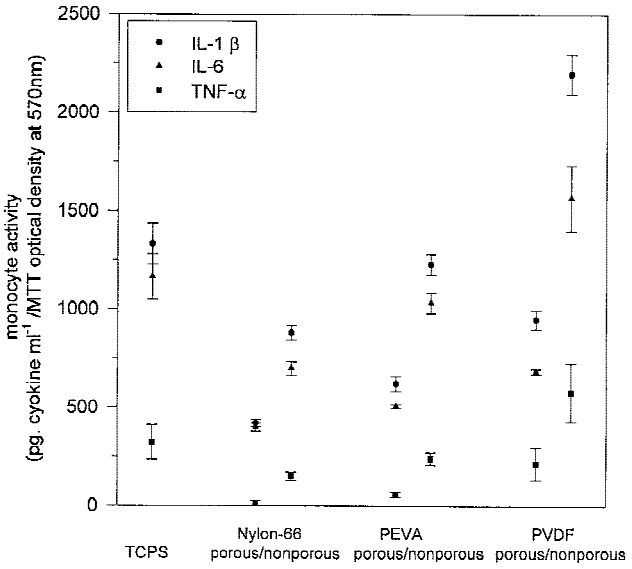


Figure 4. The monocyte activity (cytokine production/MTT conversion) on PVDF, PEVA, and Nylon-66 membranes with nonporous and porous morphologies after 24-h incubation ($n = 4$).

not influence the release of cytokines from monocytes, although they altered the ability of monocytes to attach (Fig. 3). The level of cytokine production for various membranes is similar to TCPS, except porous PEVA and porous Nylon-66 membranes showed significant inhibitory effects on TNF-α release compared with TCPS ($p < 0.05$).

We assumed the contribution of nonadherent cells to the production of cytokines is negligible. Therefore, Table I shows the cytokine production obtained only by all activated monocytes adhering to the membranes. The evaluation was also taken one step further based on the activity of individual monocytes adhering to the membranes. Monocyte activity was obtained by evaluating the production of cytokines divided by MTT conversion (i.e., the cytokine release per monocyte adhering to the membranes). This more comprehensive view of the effects of membrane surface properties in monocyte activity is presented in Figure 4. It shows the lower monocyte activity of the porous membranes and significant differences compared with TCPS ($p < 0.05$), regardless of the wettability of the polymer. Furthermore, the monocyte activity in the *in vitro* monocyte culture was PVDF > PEVA > Nylon-66, regardless of the surface. Based on our water contact angle data, monocyte activity may have a direct relationship with surface wettability. As the membrane water contact angle increased, the ability of the membrane to stimulate monocyte production of cytokines increased. Therefore, the hydrophilic membrane Nylon-66 with a porous surface was the least stimulating to monocyte cytokine production when compared with all of the other membranes evaluated with nonporous or porous surface morphology.

Cell morphology

After monocytes contact biomaterials, monocytes alter their cell membrane to stabilize the cell–material interface.[21] Subsequently, monocytes undergo shape

TABLE I
Monocyte Cytokine Production (pg/mL) on PVDF, PEVA, and Nylon-66 membranes with Nonporous and Porous Morphologies ($N = 4$)

	IL-1β		IL-6		TNF-α	
Sample	Nonporous	Porous	Nonporous	Porous	Nonporous	Porous
PVDF	989 ± 45	966 ± 50	704 ± 75	696 ± 11	260 ± 65	220 ± 85
PEVA	885 ± 36	812 ± 51	744 ± 40	662 ± 13	173 ± 23	71.0 ± 17*
Nylon-66	862 ± 37	654 ± 26	685 ± 35	623 ± 32	150 ± 20	23.5 ± 18*
TCPS	861 ± 68		753 ± 75		209 ± 56	

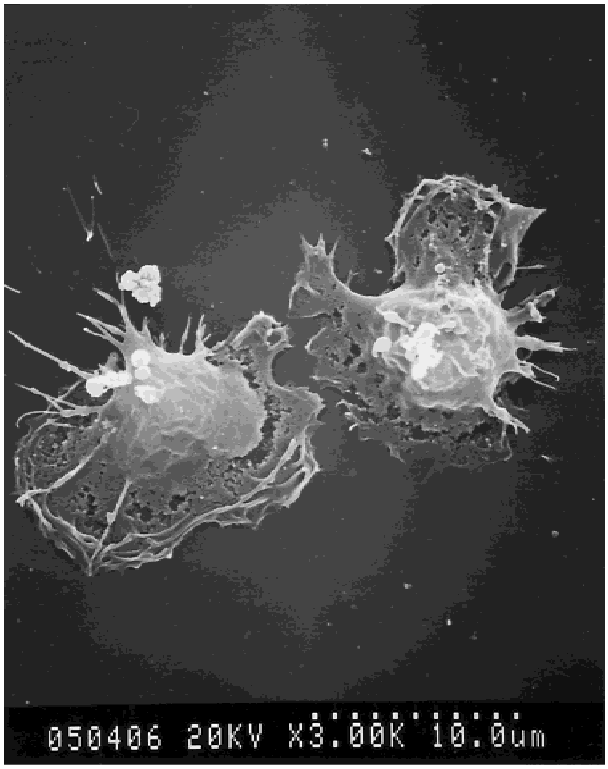
*Significant differences ($p < 0.05$) of monocyte cytokine production compared to TCPS.

changes and release of cytokines. Figure 5 shows that there were differences among the morphologies of monocytes cultured on the nonporous and porous PVDF membranes at 24 h after monocyte seeding. The nonporous membrane showed monocytes forming sheets over the surface of the PVDF [Fig. 5(a)]. However, monocytes did not spread well on the porous membrane and remained round in appearance [Fig. 5(b)]. In contrast to monocytes adherent to the porous PVDF membrane, those adherent to the nonporous PVDF membrane were of a higher activated state. The results clearly demonstrate that there were different processes during monocyte culture on membranes with different morphologies, which again confirms that the membrane morphology was an important factor for activation of monocytes as mentioned above.

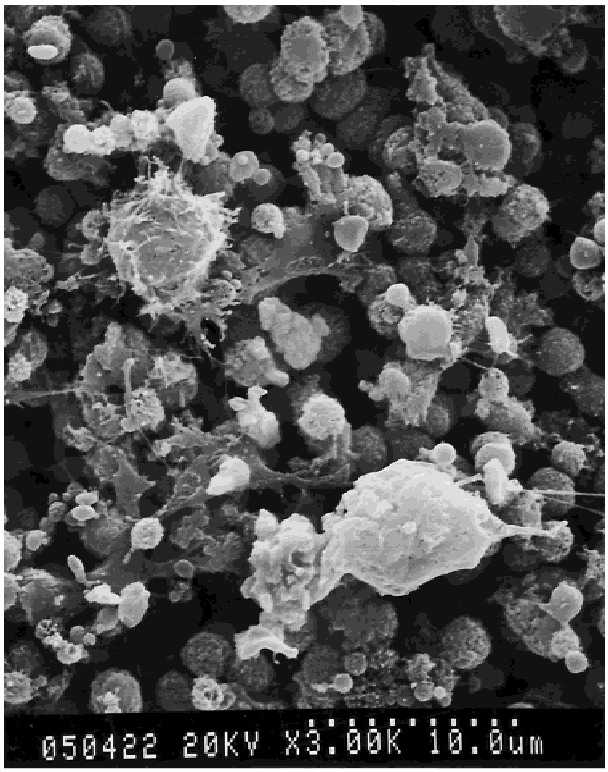
DISCUSSION

To examine the effect of surface properties of crystalline polymers on the monocyte adhesion and activation, we prepared PEVA, Nylon-66, and PVDF membranes using different preparation processes to exhibit nonporous and porous surface morphologies. Although little is known about the role of the crystalline polymer in cell–substrate interaction, it is obvious from this study that crystalline polymers with different surface morphologies and wettabilities are important in modulating monocyte adhesion and activation. When the nonporous PVDF membranes were subjected to monocytes *in vitro* for 24 h, they showed the greatest inhibition of monocyte adhesion compared with other membranes. However, they showed the greatest IL-1β, IL-6, and TNF-α secretion produced per activated monocyte. The release of cytokines by monocytes associated with biomaterials has important implications for the biocompatibility of biomaterials because these cytokines serve to activate other inflammatory cells to influence tissue response to biomaterials.[6–10] This strongly suggests that, although a biomaterial activates monocytes to augment inflammatory response, it is not certain that more monocytes are capable of adsorbing to this biomaterial. Similarly, nonporous membranes attracted fewer adherent monocytes than porous membranes whereas every activated monocyte on nonporous membranes released higher amounts of cytokine than on porous membranes.

Based on these results, although cell–substrate interaction is a dynamic and complex process, interaction of monocytes with biomaterials can be considered to be two independent processes.[22] The first process is monocyte adhesion. The second process is monocyte activation. Several studies demonstrated that extracellular matrix components have the potential to interact



(a)



(b)

Figure 5. SEM photomicrographs of monocytes adhered on (a) nonporous and (b) porous PVDF membranes after 24-h culture.

with receptor molecules in cell membranes.[23–26] For example, the presence of receptors for the Fc fragment of IgG is the criteria most widely accepted for definition of monocytes. Hence, a possible explanation for a biomaterial having different effects on the monocyte adhesion and activation is that the adhesion and activation of monocytes on a biomaterial are mediated by different ligand–receptor interactions. Presumably, the receptors are a group of surface membrane molecules that specifically recognize and bind the proteins adsorbed by the biomaterial to mediate subsequent cell behavior. Thus, proteins either residing in serum or secreted by cells play an important role in adhesion and activation of cells on substrates.[23] Colton et al. reported a suppression of IL-1 release in the presence of different coating proteins *in vitro*.[20,27,28] Hence, the different levels of IL-1β, IL-6, and TNF-α after 24 h of monocyte–biomaterial association may be related to the influence of serum in the culture medium because serum protein adsorptions by polymers may differ. Likewise, surfaces eliminating or minimizing the interactions between monocytes and adsorbed proteins would be expected to inhibit monocyte adhesion. As indicated previously, significantly less monocyte adhesion was observed on PVDF membranes but significantly more monocyte adhesion was observed on Nylon-66 membranes. This suggests the interaction between the monocyte and the adsorbed proteins on the Nylon-66 was greater than those of the PVDF. The details of indirect cell interactions with biomaterials remain poorly understood. However, in the present communication we suggest the reason for better attachment of cells on the Nylon-66 membrane was that proteins were either richly adsorbed or the adsorbed proteins took a configuration favorable for cell adhesion. In contrast, the reason for inhibited adhesion of monocytes on the PVDF membrane might be that protein-binding sites were not present or adsorbed proteins were distorted by the PVDF surface.

Moreover, it is of interest to note that monocytes showed different adhesion behavior toward the porous surface. The reason for the observation is not clear at this time. However, it is possible to attribute the difference to the contact area of the monocytes and the substrate. The contact area that monocytes adhere to on a nonporous surface is relatively small while monocytes may be embedded in a porous surface, resulting in the larger contact area. In other words, the porous surfaces can exhibit more adsorbed proteins to interact with cells. This gives rise to increases in the interactions between monocytes and substrates and then enhanced monocyte adhesion to the porous surface.

Overall, when monocytes were seeded on the substrate, monocytes underwent receptor-mediated adhesion and receptor-mediated activation. The monocyte adhered onto the substrate through the binding of the adhesion receptor to the adsorbed proteins, but the cytokine release of adherent monocytes was determined by the activation receptor. Therefore, the tendency of receptor-mediated adhesion differs from receptor-mediated activation. For example, even though a limited number of monocytes adhered to the PVDF membrane, every adherent monocyte took a configuration favorable for activation receptor-binding sites on the PVDF membrane in order to highly activate monocytes to produce a large amount of cytokines. In contrast, Nylon-66 promotes cell attachment but lacks cytokine releasing activity. Hence, the adherent monocyte on the Nylon-66 was stable and greater activation energy was required to activate the receptor of the adherent monocyte. Consequently, the development of biomaterials that eliminate monocyte activation is an approach to the preparation of biomaterials. A typical example of this type is the porous Nylon-66 membrane.

CONCLUSION

Three crystalline polymer membranes were studied as substrata for monocyte culture. The water contact angles were PVDF > PEVA > Nylon-66. For nonporous or porous surfaces the monocyte adhesion had the order of Nylon-66 > PEVA > PVDF. Monocyte activation was PVDF > PEVA > Nylon-66. The data show the correlation of membrane water contact angle on monocyte adhesion and activation. Regardless of the polymers, monocytes preferentially adhered to the porous surfaces more than the nonporous surfaces. Conversely, monocytes had rather low activity on the porous surfaces as compared to the nonporous surfaces. Monocyte adhesion and activation had opposite trends in this work, probably because different mechanisms in the cell membrane contributed to a reduction in the monocyte adhesion and an enhancement in the monocyte activation, or vice versa.

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