POLYMERS OF METHACRYLATE AVAILABLE FOR OBTAINING VARIETIES OF CELL-SUBSTRATUM ADHESIVITY

HISAO TAKAYAMA¹, TAKAHIKO TANIGAWA¹, ATSUSHI TAKAGI¹ and KOICHI HATADA²

¹Department of Bacteriology, School of Medicine, Tottori University, Yonago 683, and ²Department of Chemistry, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan

ABSTRACT

Cell attachment to chemically defined substrata was studied with mouse 3T6 cells which were seeded on glass dishes coated with syndiotactic poly(2-hydroxyethyl methacrylate), isotactic poly(ethyl methacrylate), or syndiotactic poly(ethyl methacrylate). Cells suspended in serum-free medium attached to both the isotactic and syndiotactic poly(ethyl methacrylate)-substrata but not to the syndiotactic poly(2-hydroxyethyl methacrylate)-substratum. On the other hand, cells suspended in serum-containing medium did not attach to any of the three substrata, and the inhibition of cell attachment was correlated with the dose of polymers added to the glass dishes. The dose-response experiment showed that there was a little, but significant, difference between isotactic and syndiotactic poly(ethyl methacrylate)s. The results indicate that the substitution of hydrogen for the hydroxyl group in poly(2-hydroxyethyl methacrylate) results in notable modifications of substratum adhesivity; the inhibition of cell attachment to poly(2-hydroxyethyl methacrylate) is independent of serum components whereas the inhibition of cell attachment to poly(ethyl methacrylate) is dependent on serum components, and syndiotactic poly(ethyl methacrylate) more sharply disturbs cell attachment than does the isotactic polymer.

Cell attachment to appropriate substrata is profoundly associated with the physiological conditions of the cells such as growth (25) and viability (21). Substrata modified with poly(2-hydroxyethyl methacrylate) (PHEMA) and poly(methyl methacrylate) (PMMA) have been widely utilized in various experiments to obtain desired adhesivity for cell attachment. These studies included investigations on cell growth (1, 4, 7, 25), macromolecular metabolism (2), and expression of a differentiated property (3, 18). Their aim was to find the desired conditions for substratum (anchorage) efficiency which is controlled by the dose of PHEMA.

In this study, three species of polymethacrylate, *i.e.* syndiotactic poly(2-hydroxyl methacrylate) (syn-PHEMA), syndiotactic poly(ethyl methacrylate) (syn-PEMA) and isotactic poly-

(ethyl methacrylate) (iso-PEMA) were used. The principal aim of this study was to determine the availability of the three polymers and to investigate the different responses of cultured cells, mouse 3T6 cells, which are well characterized. To the best of our knowledge, this type of approach has never been tried. It was found that the substitution of hydrogen for the hydroxyl group and the changes in tacticity in polymethacrylate results in notable modifications of substratum adhesivity.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The 3T6 mouse fibroblast cell line (28) was used in all the experiments. The cells were subcultured in Dulbecco's modified Eagle's medium

with 0.1% glucose (DEM) (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Control No. 27P8108), penicillin G (500 U/ml) and streptomycin (100 μ g/ml) in 35 $\times$ 10 mm or 60 $\times$ 15 mm plastic dishes (Falcon Labware, Div. Becton, Dickinson Overseas, Oxnard, CA, U.S.A.; Catalogue No. 3001 or No. 3002) at 37°C in a humidified incubator flushed with a CO₂-air mixture. Cells grown to subconfluent density were dispersed with 0.05% trypsin (Difco Laboratories, Detroit, MI, U.S.A.) and 0.02% ethylenediamine tetraacetate (EDTA) (Nakarai Chemicals, Kyoto, Japan) in phosphate-buffered saline free of magnesium and calcium at 37°C for 10 min, and resuspended in DEM containing or lacking fetal calf serum when assayed.

Preparation of Poly(Ethyl Methacrylate)s

Ethyl methacrylate (EMA) was purified by the usual manner, dried over calcium dihydride, and distilled under high vacuum just before use. Polymerization was carried out in a flame-dried glass ampoule under a nitrogen atmosphere. Iso-PEMA was obtained by polymerizing the monomer with 3 mol% of butyllithium in toluene at -78°C for 24 h (15). The reaction mixture was poured into a large amount of methanol and the insoluble syndiotactic polymer was removed by centrifugation. The soluble fraction was recovered by evaporating the methanol solution. The iso-PEMA thus obtained was purified by freeze-drying. The number and weight-average molecular weights determined by gel permeation chromatography (GPC) were 10,600 and 534,800, respectively.

Syn-PEMA was obtained with 1 mol% of 2,2'-azoisobutyronitrile in toluene at 60°C. The methanol-insoluble polymer was collected by filtration and dried under vacuum at 50°C. The number and weight-average molecular weights determined by GPC were 65,800 and 104,200, respectively. Triad tacticities of the isotactic and syndiotactic PEMAs were determined by ¹H-NMR spectroscopy as follows:

iso-PEMA: I=77.3, H=13.4, S=9.3%

syn-PEMA: I=12.6, H=29.3, S=58.2%

Coating of Glass Dishes with Methacrylate Polymers

The syn-PHEMA was dissolved in ethanol (Wako Pure Chemical Industries, Tokyo, Japan), and the iso- and syn-PEMA's were dissolved in

toluene (Wako Pure Chemical Industries) at a concentration of 20 mg per ml at 55°C for 24 h. These stock solutions were diluted with ethanol or toluene from 20 mg/ml to 20 μ g/ml at 55°C. One-hundred-microliter samples of the warmed solution were pipetted into clean glass dishes (Flat, Nagareyama, Chiba, Japan; Catalogue No. P-1, 9.58 cm²) and the solution was spread on the bottom surface of dishes, with small bent glass rods if necessary, and the coated dishes were dried immediately on a level tray in an incubator with gentle ventilation at 55°C for 24 h. Before use, the dishes were exposed to a 15-W germicidal ultraviolet lamp at a distance of 60 cm for 60 min to sterilize the bottom surface.

Assay for Cell Attachment

Cell attachment was determined by a shaking method as described previously (19). Briefly, harvested cells were washed once, and 5.0×10^5 cells were resuspended in 2.0 ml of DEM, seeded on the dishes precoated with polymers, and incubated in the CO₂-incubator at 37°C. After incubation, the dishes were shaken at room temperature and the medium was discarded. The cells considered to be unattached were removed by this procedure. The percentage of attached cells was calculated as the number of cells remaining on the bottom surface divided by the total number of cells seeded.

Assay for Cell Spreading and Cell Height

The extent of cell spreading (area covered with cells) was measured microscopically as described previously (19). Cell height was measured by the method described by Folkman and Moscona (7).

Pretreatment of Substratum with Serum

In some cases, substrata to be used for assaying cell attachment were pretreated by incubating glass dishes coated with polymers in the CO₂-incubator with 2 ml of DEM containing 10% fetal calf serum at 37°C for the chosen periods. The dishes were rinsed well with serum-free DEM, and then used for the assay.

RESULTS

The time courses of cell attachment to the polymer substrata and the glass substratum (control substratum) in the presence and the absence of serum are shown in Fig. 1. Most of the cells attached to the glass substratum by 6 h of incuba-

Fig. 1 Time course of cell attachment to polymer-coated dishes in the presence or absence of serum. 3T6 cells were grown to subconfluence, dispersed with trypsin and EDTA, resuspended in DEM containing or lacking serum (10%), seeded in dishes coated with 2 mg of polymer per 35-mm dish at a cell density of 5.0×10^5 cells/dish, and then incubated in a CO_2 -incubator at 37°C . Cells attached to the dishes were counted after the dishes were shaken to remove the unattached cells. Each point in the figure represents the mean of values from two cultures (vertical bars).

tion, though the addition of serum resulted in a lag phase in cell attachment. In the presence of 10% serum in the medium, cells did not attach to the polymer substrata. On the other hand, in the absence of serum, cells did attach to the dishes coated with 2 mg of iso-PHEMA per 35-mm dish, but not to those coated with syn-PHEMA (Fig. 1). The final extent of attachment to the iso-PHEMA substrata at 6 h of incubation was slightly lower than the maximum value at 2 h. No differences were observed between the uncoated dishes (glass substratum) and the substrata pretreated with ethanol or toluene as solvent (data not shown). The results with syn-PHEMA-coated dishes were very similar to those with iso-PHEMA-coated dishes (data not shown).

The effect of polymer dose on cell attachment was studied in the presence of 10% serum. The results are shown in Fig. 2. The extent of cell attachment decreased with increasing doses of the polymers. The dose-dependent inhibition of cell attachment indicates that the polymers are a primary cause of the inhibitory effect. It should be noted that there was a little but measurable difference in inhibitory efficiency between iso-PHEMA and syn-PHEMA; syn-PHEMA was more inhibitory than iso-PHEMA.

In the presence of 10% serum, cell spreading was inhibited by the higher doses of syn-PHEMA as shown in Fig. 3. Similar results were obtained in the experiment with iso-PHEMA (data not shown). The cell spreading was inhibited by syn-

Fig. 2 Effect of polymer dose on cell attachment. Glass dishes were coated with various doses of the three polymers. 3T6 cells were seeded in the coated dishes at a cell density of 5.0×10^5 cells/dish and incubated at 37°C for 6 h. Cells attached to the dishes were counted as described in the legend for Fig. 1. Each point in the figure represents the mean of values from two cultures (vertical bars).

Fig. 3 Effect of polymer dose on cell spreading. Glass dishes were coated with the polymers, and cells were seeded in the dishes and incubated for 6 h under the conditions given in the legend for Fig. 2. Cells were photographed under a phase contrast microscope. The final magnification, $\times 100$

Fig. 4 Effect of polymer dose on both cell spreading and cell height. Cells were prepared under the conditions described in the legend for Fig. 2. Cell height and cell spreading were measured as described in Materials and Methods. Each point represents the mean $\pm$ SD of at least 50 cells from two cultures.

PEMA in a dose-dependent manner (Fig. 4). The dose response curve of cell spreading was roughly parallel to that of cell height, indicating that inhibition of cell spreading was associated with inhibition of cell flattening or reduction of cell height (Fig. 4). These results suggest that cell shape (spreading or height) is controlled by the graded adhesiveness of the different dose of polymers (Figs. 2 and 4).

The effect of serum concentration on cell attachment to the dishes coated with 2 mg of polymers per 35-mm dish was studied (Fig. 5). Hardly any cells attached to the dishes coated with syn-PHEMA whereas the majority of cells attached to the glass substratum. This phenomenon was independent of the serum concentrations. However, cell attachment to the syn- or iso-PHEMA-substrata was inhibited by 2% or higher concentrations of serum; that is, the inhibition of cell attachment by these polymers was dependent on the concentration of serum in the medium.

A study was undertaken to determine whether the serum was adsorbed into the substratum. Conditioning substrata pretreated with 10% serum in the medium were used in the fol-

lowing experiments. The adhesiveness of the glass substratum and of the syn-PHEMA-substratum was barely modified by the pretreatment with the medium containing serum. However, the iso- and syn-PHEMA-substrata were affected by the pretreatment; pretreatment for at least 60 min resulted in the inhibition of cell attachment to both the iso- and syn-PHEMA-substrata. This inhibitory effect of pretreatment with serum is probably due to the adsorption of serum into the substrata (Fig. 6).

DISCUSSION

Evidence from the present study suggests that the inhibition of cell attachment is brought about by two different mechanisms; one occurs in a serum-independent manner on the PHEMA-substratum, the other occurs in a serum-dependent manner on the PEMA-substratum. It is also revealed that the inhibition of cell attachment is controlled by the dose of these polymers. Thus, the cell attachment to a substratum can be modified by using these chemically defined polymers. We will discuss some aspects of these available polymers.

Fig. 5 Effect of serum concentration on cell attachment to dishes coated with the polymers. 3T6 cells were resuspended in DEM containing various concentrations of serum, seeded in dishes coated with 2 mg of polymer per 35-mm dish at a cell density of 5.0×10^5 cells/dish, and incubated at 37°C for 6 h. Cells attached to the dishes were counted. Each point in the figure represents the mean of values from two cultures (vertical bars).

Fig. 6 Effect of pretreatment of dishes on cell attachment. The dishes were pretreated with serum-containing DEM (see Materials and Methods) and rinsed well with serum-free DEM. 3T6 cells were resuspended in serum-free DEM, seeded in the pretreated dishes at a cell density of 5.0×10^5 cell/dish, and incubated at 37°C for 6 h. Cells attached to the dishes were counted. Each point in the figure represents the mean of values from two cultures (vertical bars).

First, the inhibition of cell adhesivity to dishes coated with the polymers is not a cause of sublethal cytotoxicity on the part of the polymers, because the possibility of toxic effects was ruled out by two experiments. 1) Conditioning medium was prepared by incubating DEM containing fresh 10% fetal calf serum in polymer-coated glass dishes at a dose of 2 mg/35-mm dish at 37°C for 3 days. No reduction of plating efficiency of 3T6 cells was ever found in the conditioned medium from either polymer-coated dishes or uncoated dishes (data not shown). 2) Deep scratches were made in thick polymer-films on glass dish substrata. Cells attached, spread, and proliferated normally on the scratched area surrounded by polymer-films, but not on the films (data not shown). Folkman and Moscona have already reported that PHEMA is nontoxic (7).

Second, our studies were carried out with cells dispersed with trypsin and EDTA. It must be considered that cell damage can occur during the dispersion. However, it has been reported that the effect of dispersion can be reversed for a short time (16), and cell attachment is barely affected by the dispersion treatment (10). Although we did not carry out control experiments with non-dispersed cells, the effect of the dispersion may be negligible, at least under the present experimental conditions.

Third, the molecular mechanisms of cell attachment to the substrata described here remain unclear, but several explanations can be posed from the results of the present study by comparing the three polymers. The *in vitro* process of cell adhesion and spreading consists of four events, 1) attachment of cells at the point of contact with the substratum, 2) centrifugal growth of filopodia, 3) cytoplasmic webbing, and 4) flattening of the central mass (24). Firm attachment to the substratum requires the expenditure of metabolic energy (9, 22). Serum contains cell attachment-inhibiting factors (27) such as albumin (12), and also contains cell attachment-enhancing factors (11, 13) such as fibronectin (6, 14). In addition, collagen, which can bind with the serum factors, has important roles in cell attachment (6, 14, 20, 23). Yamada and Olden have reviewed these adhesion-associated proteins (30). On the other hand, it has been reported that 3T6 cells, used in the present study, have a capacity for producing fibronectin (17, 29), and collagen (8). In the present work it was found that cells did not attach to the PHEMA-substratum even in the absence of serum. This suggests that 1) cells have no binding site for the PHEMA-substratum and/or 2)

cell attachment is interfered with by the surface nature of PHEMA in the early stage of cell approach. The fact that cells could attach to PEMA but not to PHEMA in the absence of serum suggests that hydroxyl groups in the polymer molecules play an important role in the inhibition of cell attachment. Furthermore, the findings that the cells could attach to the PEMA-substratum in the absence of serum but not in its presence indicate that the effects of cell attachment-inhibiting factors in the serum overcome their counterparts. There are two possible interpretations 1) that the PEMA-substratum has a higher affinity for cell attachment-inhibiting factors such as albumin than attachment-promoting factors such as fibronectin and collagen; 2) that both factors are modified by the binding to the PEMA-substratum. On the other hand, there are many differences in the surface properties of PHEMA and PEMA including water content, interfacial free energy and surface charge (5, 26). In any case the interaction between the polymer-substratum and the cells or serum components strongly depends on the chemical and steric structure of the polymer. However, a definitive conclusion must await the outcome of more detailed analysis.

Finally, PHEMA controlling cell adhesivity has been extensively used (1-4, 7, 18), and the polymers have already provided a fundamental procedure for demonstrating the mechanisms of cell adhesion. We believe that, in addition to PHEMA, iso- and syn-PEMAs serve well as useful tools for evaluating the cell response to a substratum as demonstrated in this study.

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