Comparative study of human monocyte and platelet adhesion to hydrogels *in vitro*–effect of polymer structure

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Blood platelet and monocyte adhesion was studied *in vitro* with respect to the influence of hydrophilic polymer chemical functional groups and their charge. The results showed that the strongest adhesion of human monocytes was to coverslips covered with cationic polymer. Platelet adhesion to all tested polymers proved to be negligible; no differences related to the charge of the polymers used were observed. These results show the obvious difference between the biocompatibility and haemocompatibility *in vitro* which must be taken into consideration during polymer biological properties testing before clinical trials.

1. Introduction

Non-activated platelets and monocytes are free-floating cells in blood. Adhesion of these elements to the solid substrate represents a crucial condition of their interaction with synthetic polymers and plays an important role in the biocompatibility of implanted devices [1]. It is generally known that cell adhesion to the solid support is a very complicated, complex process, including: (1) adsorption of proteins from blood plasma or tissue fluids; (2) specific recognition of these proteins by cell surface receptors; (3) nonspecific interaction of cell surface molecules (namely oligosaccharides) with adsorbed proteins and implant material. The adhesion and level of eukaryotic cell spreading seemed to be responsible for the specific gene activity control [2] and platelet activation [3].

Synthetic, crosslinked polymers are not directly and specifically recognized by cells owing to the absence of receptors for these macromolecules. These materials, however, induce protein adsorption, different in quantity and protein conformation with respect to the chemical structure of polymers [4]. From this point of view, hydrophilic polymers (hydrogels) have specific properties. They induce only low surface protein adsorption [5] and they are known as only poorly adhesive and thrombogenic materials [6]. On the other hand, previous results clearly demonstrated that the molecular design of hydrogels affects the adhesivity, spreading and fusion of macrophages, colonizing the surface of the implanted hydrogel. The level of macrophage response is dependent on the occurrence of functional chemical groups in the polymer (dimethylamino-, hydroxy-, carboxy-) [7–10]. In this study we compared the adhesivity of freshly isolated human monocytes and platelets to hydrogels, with respect to polymer design and protein preadsorption *in vitro*. The markers of cell differentiation were studied immunocytochemically.

2. Materials and methods

2.1. Polymers

Polymers poly (2-hydroxyethylmethacrylate) [poly HEMA], poly (diethyleneglycolmethacrylate) [poly DEGMA] and statistical copolymers of 2-hydroxyethylmethacrylate with methacrylic acid [poly HEMA-*co*-MAA] and 2-hydroxyethylmethacrylate with dimethylaminoethylmethacrylate [poly HEMA-*co*-DMAEMA] (97:3, 90:10 mol % resp. in the polymerization mixture) were prepared using a polymerization solution in ethanol (1:10 w/v), using azobis-isobutyronitrile (0.1% relative to monomer weight) as an initiator (70 °C, 4 h). Polymers and copolymers were precipitated from the reaction mixture into petroleum ether and purified by reprecipitation from ethanolic solution.

For further investigation, the 7.5 wt % solution of polymeric samples in methylcellosolve were spin cast onto clean microscopic coverslips (20 × 20 mm) and further air-dried in a laminar box.

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2.2. Human monocyte preparation and adhesion assay

Monocytes were isolated from 10 ml of peripheral heparinized blood (10 U/ml), using Percoll-gradient centrifugation. The cells were washed twice in a minimal essential medium (MEM) and resuspended in 0.5 ml MEM. Pooled serum, heat inactivated serum (56 °C, 60 min) or plasma was added to the cell suspension to obtain the final serum (or plasma) concentration of 10% (v/v). The cell number was adjusted to 10^8/ml.

Pure glass coverslips and coverslips with experimental polymers (poly HEMA, poly HEMA-*co*-MAA, poly HEMA-*co*-DMAEMA) were preincubated with MEM for polymer layer swelling. Monocyte suspension (0.1 ml) was applied to a coverslip and incubated in humid atmosphere and 5% (v/v) CO_2, for 90 min.

The coverslips were washed with PBS, fixed with 1% paraformaldehyde and stained for acid phosphatase (AcP) and CD-14 antigen detection. A mouse monoclonal antibody (MEM-15, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague) was used as a first-step antibody and the peroxidase conjugated swine polyclonal anti-mouse immunoglobulins (SwAM-Px, SEVAC, Prague) as the second one, with diaminobenzidine (Sigma) as the chromogen. The reaction specificity was tested by sample incubation with non-immune mouse serum. The number of adhered cells was calculated oculometrically in 0.2 mm^2 and determined in three independent experiments.

2.3. Platelet adhesion assay

2.3.1. Static adhesion

Platelet adhesion experiments were performed in a six-well plate (Nuclon, Intermed). Glass coverslips coated with polymer were incubated with whole citrated blood (1 ml/well) on the circular shaker (IKA – Schüttler MTS4) at room temperature and a shake speed of 150/min, for 10 min. Blood was then aspirated, the coverslips washed with PBS and then fixed with 1.3% paraformaldehyde.

2.3.2. Perfusion experiment

Perfusion was carried out in a parallel plate perfusion chamber under well defined flow conditions [11]. The polymer (or protein) coated coverslip was inserted in the perfusion chamber and the system was perfused with whole citrated blood at wall shear rate of 300 s^{-1}, for 5 min at 37 °C. Subsequently, 25 ml of prewarmed PBS was drawn through the system, to wash the coverslip. The coverslip was then removed, fixed for 5 min in 1.3% paraformaldehyde and stained.

Before and after perfusion, samples of blood were taken to measure platelet count and free haemoglobin concentration. Platelet count was reduced during perfusion by 14 $\pm$ 4%. Free haemoglobin concentration increased from the normal range of 54 $\pm$ 15 mg/l to 200 $\pm$ 80 mg/l. Haemolysis, due to the roller pump, was thus not greater than 0.1% of total haemolysis.

2.3.3. Platelet staining

Coverslips were stained by May-Grünwald-Giemsa or immunohistochemically. Monoclonal antibodies to platelet glycoprotein IIIa (Dako), GMP 140 (Immunotech) and an antibody to fibrinogen and fibrin D-domain (85D4, Sigma) were used as first-step antibodies, and SwAM-Px (SEVAC, Prague) as the second one.

2.3.4. Fibrinogen adsorption

Fibrinogen was adsorbed to the coverslips (pure glass or polymer coated) from a 100 μg/ml solution in TRIS-buffer (50 mM TRIS, 0.1 M NaCl, pH 7.4) at RT for 1 h. Coverslips were then blocked with 1% bovine serum albumin in TRIS-buffer.

The amount of fibrinogen adsorbed to the surface (hydrogels, glass) was determined by radioactively labelled fibrinogen. Fibrinogen was labelled with ^{131}I, using Iodogen (Pierce, USA) [12a].

3. Results

3.1. Monocyte adhesion

Application of different monocyte cultivation conditions, when using plasma, serum and heat inactivated serum, only slightly influenced monocyte adhesion to the various surfaces. Use of heat-inactivated serum insignificantly decreased the number of CD14 and AcP positive monocytes adhered to all tested materials.

Poly HEMA-*co*-DMAEMA induced adhesion of both CD14 and AcP positive cells to the greatest

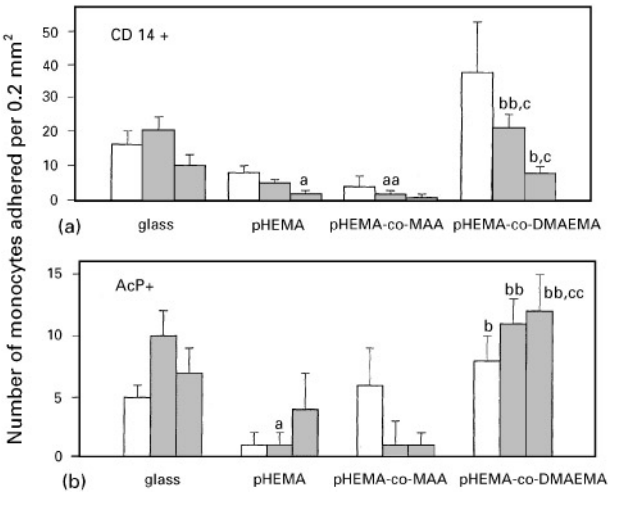

Figure 1 Monocyte adhesion to glass and hydrogels (poly HEMA, poly HEMA-*co*-MAA, poly HEMA-*co*-DMAEMA) under different monocyte cultivation conditions – using plasma (■), serum (□) and heat inactivated serum (▩). The number of monocytes positive for CD 14 (a) and AcP (b) was calculated in 0.2 mm^2; values obtained from three independent experiments are mean $\pm$ SEM. Significant differences: from glass (a: $p \leq 0.1$, aa: $p \leq 0.05$), from poly HEMA (b: $p \leq 0.1$, bb: $p \leq 0.05$), from poly HEMA-*co*-MAA (c: $p \leq 0.1$, cc: $p \leq 0.05$).

extent (21 ± 7 resp. 11 ± 3 cells; plasma cultivation conditions). Monocyte adhesion to glass was essentially the same (21 ± 4 CD14 positive cells, 10 ± 2 AcP positive cells; plasma). Significant reduction in monocyte adhesion to poly HEMA (5 ± 1 CD14 and 1 ± 1 AcP positive cells) and poly HEMA-*co*-MAA (2 ± 1 CD14 positive cells, 1 ± 2 AcP positive cells) was observed (Figs 1 and 2).

The total amount of adhered cells positive for AcP was, in general, lower than for CD14 (see Fig. 1).

3.2. Platelet adhesion

In both static and flow experiments similar results were obtained. Single dendritic and spread platelets adhered to immobilized fibrinogen and to pure glass

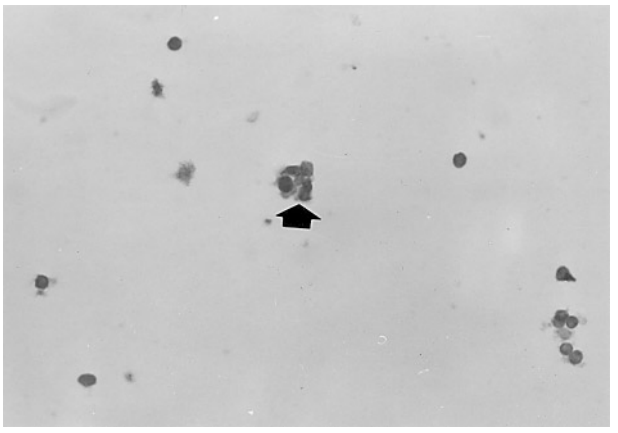

Figure 2 Adhesion of human mononuclear cells to glass coverslip. Stained for CD 14 antigen detection. Original magnification 80 x.

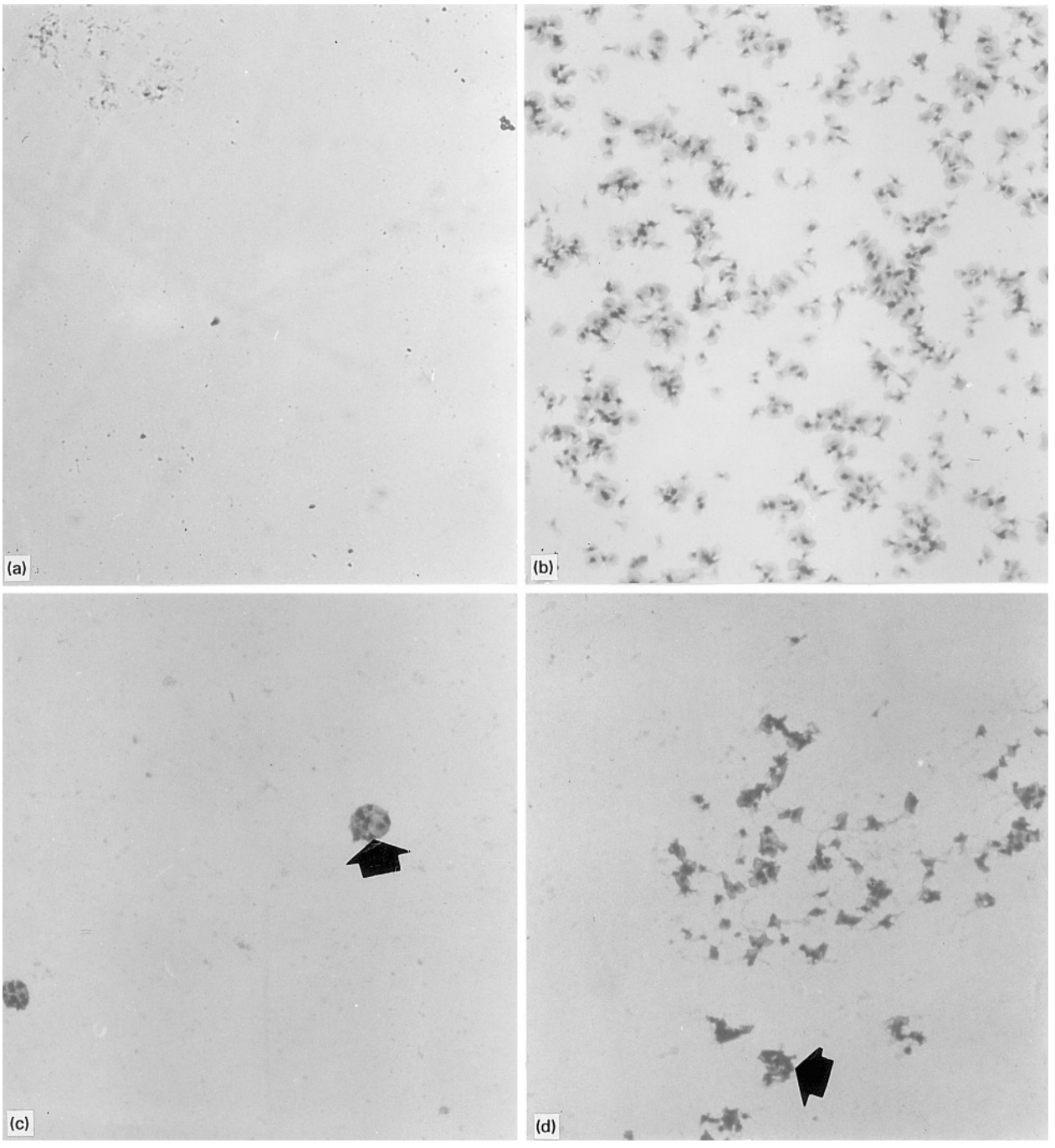

Figure 3 Platelet adhesion to coverslips coated with albumin (a), fibrinogen (b), poly (HEMA) (c) and fibrinogen preadsorbed poly (HEMA) (d) at static conditions. Platelet fragments are designated with arrows. Original magification 80 x.

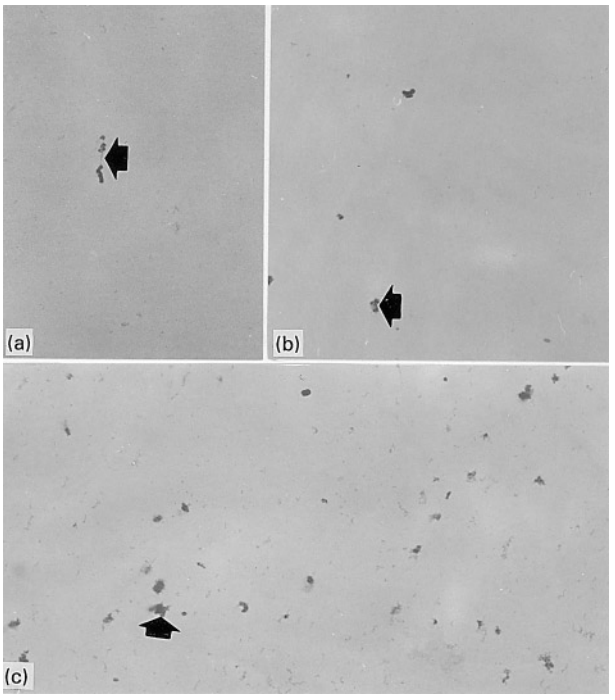

Figure 4 Platelet adhesion to poly (HEMA- *co*-DMAEMA) (a) and poly(DEGMA) at static conditions. Detected using MoAbs to GP IIIa (a), fibrin(ogen) D domain (b) and GMP–140 (c). Original magnification of 80 x.

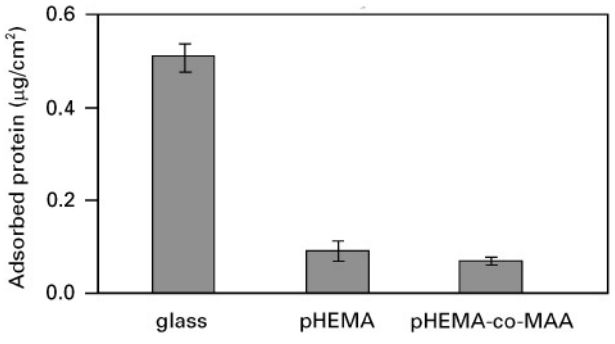

Figure 5 Fibrinogen adsorption to glass and coverslips coated with hydrophilic polymers. Protein was adsorbed from 100 μg/ml ^{131}I-labelled fibrinogen solution.

coverslips. When coverslips were pretreated with albumin, adhesion of platelets was negligible. Almost no platelet adhesion to the tested hydrogels, i.e. poly HEMA, poly HEMA-*co*-MAA, poly HEMA-*co*-DMAEMA and poly DEGMA, occurred (Fig. 3). Only platelet fragments with a tendency to cluster, were observed on the surfaces. These fragments were positive for GP IIIa, GMP 140 and fibrinogen/fibrin D domain, similar to the platelets adhered to pure glass coverslips (Fig. 4). The pre-incubation of polymer-covered coverslips with fibrinogen clearly induced the adhesion of platelets (Fig. 3d).

3.3. Adsorption of fibrinogen

The adsorption of radioactively labelled fibrinogen to coverslips coated with tested polymers, was significantly lower than that to glass coverslips (Fig. 5, $p < 0.001$).

4. Discussion

The cationic poly HEMA-*co*-DMAEMA induced the highest level of human monocyte adhesion. Adhesion of these cells to poly HEMA and/or poly HEMA-*co*-MAA was significantly decreased; the difference in monocyte adhesion to these two copolymers was only minimal. An increased leukocyte adhesion to positively charged supports in tissue culture has also been observed by others [12, 13]. In accordance with *in vitro* experiments, after implantation in rats, the poly HEMA-*co*-DMAEMA induced greater macrophage adhesion and fusion than poly HEMA or poly DEGMA [7–10]. In contrast, implants containing 3 wt % of methacrylic acid or sodium methacrylate supported the adhesion of macrophages only to a small extent and no multinucleate cells were observed on their surface [7–10]. Not only the polymer charge and structure, but also monocyte cultivation conditions influenced their adhesion to hydrogels. The present experiments have shown an insignificant decrease of monocyte adhesion to tested hydrogels when using inactivated serum.

In static and flow experiments, similar to the results of Groth and coworkers [16], no difference in platelet adhesion to hydrogels was observed. Platelets did not adhere to all tested polymer surfaces. Hydrophilic surfaces, namely negatively charged, are generally known as materials with very low *in vitro* platelet adhesivity [17–26]. This phenomenon is particularly influenced by the low fibrinogen adsorption to their surface, also observed in this study [5, 27–29]. In the current experiments, polymer charge did not affect platelet adhesion. Cell fragments on hydrogels are probably remnants of damaged platelets because they express typical platelet surface (GP IIIa) and activation (GMP 140) markers. Platelet fragmentation on hydrophilic surfaces was observed by Ratner's group [24]. At present the explanation of this phenomenon is only hypothetical. Probably this is caused by platelet damage during detachment from the poorly adhesive support.

In conclusion, monocytes *in vitro* adhered very strongly to positively charged polymers. On the other hand, platelet adhesion to all hydrophilic supports was only minimal and was not influenced by the polymer charge. These results indicate the obvious difference between the interaction of monocytes and blood platelets *in vitro* with respect to synthetic polymer structures.

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