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Macrophage recognition of polymers: effect of carboxylate groups

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The effect of anionic carboxylate groups in copolymers of 2-hydroxyethyl methacrylate with sodium methacrylate on the adsorption of albumin, adhesion, spreading and fusion of macrophages has been demonstrated. The results show an inhibitory effect of increasing concentration of carboxylate groups of polymers on all studied parameters.

1. Introduction

The interaction of an organism with implanted foreign materials is a very complex process comprising the adsorption of plasma and/or tissue fluid proteins on the implant surface and the subsequent interaction of surrounding cells and tissues with the implant [1, 2]. Implanted synthetic polymers are recognized as non-self (although they are not antigenic from the point of view of classical immunology), and macrophages are of a great importance in the control of the response of an organism to them [3]. However, knowledge of the influence of the chemical structure of polymers on macrophage-implant recognition is only minimal.

The effect of negatively charged carboxylate groups of the copolymer 2-hydroxyethyl methacrylate (HEMA)-co-sodium methacrylate (NaMA) (in a defined molecular ratio) on the serum albumin adsorption and macrophage adhesivity *in vitro*, has been investigated. The spreading of macrophages and their subsequent fusion into foreign-body giant multinucleate cells was evaluated 9 days after implantation in the rat with respect to the concentration of carboxylate groups in the polymers.

2. Material and methods

2.1. Polymers

Poly HEMA and copolymers HEMA-co-NaMA with 1, 2 and 3 mol % NaMA have been prepared by polymerization in an inert nitrogen atmosphere between two heated plates at 60 °C. Other components of the polymerization mixture were glycerol (environment) 20 wt %, 2-hydroxyethyl dimethacrylate (cross-linking agent) 0.3 wt % and sodium 2,2′-azo*bis* (2-methyl propionitrile) (initiator) 0.1 wt %.

Polymers and copolymer samples 0.7–0.9 mm thick, were converted with 0.06 mol % solution of sodium bicarbonate into the sodium-form, sterilized (20 min, 120 °C) and kept in a physiological solution.

2.2. Human serum albumin (HSA) adsorption

2.2.1. Photometric procedure [4]

Polymeric strips were incubated in PBS (0.01 M phosphate buffer, pH 7.2, 0.14 M NaCl) with human serum albumin (HSA) fraction V (SIGMA, St. Louis, USA) in a concentration 1 mg HSA/ml at 37 °C for 60 min. The samples were washed for 60 min with PBS and stained by Coomassie Blue R (Serva, Heidelberg, Germany) for 40 min. The adsorbance was measured at 540 nm. The HSA deposition was estimated from the difference of the absorbance of polymers incubated with HSA and of unincubated but also stained samples.

2.2.2. Procedure with radiolabelled HSA

HSA (protein purity better than 99%, Institute of Sera and Vaccines, Olomouc, CR) was radiolabelled with ^{125}I using the Bio-Rad Enzymobead method. Briefly, 50 µl dehydrated Enzymobead reagent (Bio/-RAD, Richmond, USA), 50 µl 2% solution of HSA in PBS, 10 µl $Na^{125}I$ (1 mCi, Institute of Nuclear Research, Řež, CR) and 25 µl 1% β-glucose in PBS were mixed and reacted for 30 min at room temperature. The labelled HSA was separated chromatographically on a minicolumn with Sephadex G 25M (Pharmacia, Uppsala, Sweden). The yield was 1.5 ml HSA solution containing 0.45 mg HSA with the specific activity 0.1 mCi/1 mg HSA, the ratio of unbound to bound iodine, was smaller than 5×10^{-3}. The labelled HSA was used for no longer than 2 days after labelling. Solutions of labelled and unlabelled HSA were mixed before the adsorption experiment to contain 1.5 mg HSA/1 ml with a specific activity about 5 µCi/mg HSA.

The polymeric samples were conditioned for 24 h in PBS, then the solution of labelled HSA was added so that the final solution contained 1.0 mg HSA/1 ml. The samples were immersed during the adsorption, and the system was gently mixed. After 60 min adsorption at 25 °C, the HSA solution was replaced with

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PBS. Washing and desorption followed for 60 min. The amount of adsorbed HSA was determined from the radioactivity of samples measured by a well-type of γ-scintillation counter (Tesla, CR).

2.2.3. *X-ray photoelectron spectroscopy (XPS)*

The surface elemental analysis (except of hydrogen) of samples was carried out with ESCA 3 Mark II (VG Scientific Instruments, UK) using the Al_α source of X-rays and passing energy 50 eV. The XPS spectra were measured at an output angle of photoelectrons 55°, the depth of surface analysis thus being about 8.5 nm.

The relative amounts of HSA adsorbed on the sample were estimated from the nitrogen contents. Scofield's values of cross-sections were used in the calculations.

2.3. Macrophage *in vitro* adherence under serum-free conditions

Rat peritoneal macrophages were harvested according to Lentz *et al.* [5]. The collected cells were centrifuged and washed in cold PBS at 4 °C. This procedure was repeated three times.

The samples of polymers were incubated with cells (seeding density 220 cells mm^{-2}) at 37 °C for 10 min. After PBS washing and 4% paraformaldehyde fixation, the number of cells adhering to 1 mm^2 polymers was calculated using phase contrast microscopy.

2.4 *In vivo* study in laboratory rats

Five strips of each polymer were subcutaneously implanted in laboratory rats (Wistar, Velaz, Prague, CSFR) of both sexes as described [6]. The implants were removed 9 days after surgery. The strips were cut in two parts. The first was fixed by Baker's fixative for 5 min and stained by haematoxylin and eosin. These samples were used in a study of the cytological appearance of the implant surface with the main aim at estimating the fusion ability of macrophages, expressed by the fusion index *FI* [7, 8]: FI ± SD = number of nuclei in multinucleate cells/number of nuclei in mononuclear macrophages and in multinucleate cells. The level of mononuclear macrophage spreading was measured by means of an ocular lattice in at least 100 cells on the surface of each sample. The second part of each strip was fixed with 2% glutaraldehyde and stained with Ruthenium Red (Sigma, St. Louis, USA) according to Kashiwa *et al.* [9] for anionic group detection. The stained and washed specimens were routinely dehydrated and embedded in Epon 812 (Serva, Heidelberg, Germany). The semi-thin sections (Ultracut, Reichert, Wien, Austria) were stained by Toluidine Blue. The ultra-thin sections (Ultracut, Reichert, Wien, Austria) were observed without contrast in a Jeol JEM-200 CX electron microscope, including the X-ray detection of Ruthenium specific spectra.

3. Results

3.1. Albumin adsorption

All analytical procedures used demonstrated the inhibitory effect of carboxylate groups on the HSA adsorption on to polymers (Table I).

3.2. *In vitro* macrophage adherence study

All samples showed a low adhesivity of macrophages and a negative effect of carboxylate groups on their adhesion (Table II).

3.3. *In vivo* study in laboratory rat

The surface of the polymers was colonized by typical cells of foreign-body reaction, i.e. by macrophages, foreign-body giant multinucleate cells and occasional polymorphonuclears, lymphocytes and plasma cells (Figs 1 and 2). However, the cellularity, the number and size of multinucleate cells, were negatively influenced by the increasing concentration of carboxylate groups. Similarly, the spreading of macrophages was also inversely dependent on the carboxylate groups concentration (Figs 1 and 2, Table II).

The thin layer of electron-dense Ruthenium Red was detected on the macrophage and on the giant multinucleate cell surface including the cell–implant interface (Fig. 3). A small amount of adsorbed protein between the cell and polymer surface was also observed (Fig. 3).

4. Discussion

The results demonstrate the influence of the concentration of carboxylate groups on albumin adsorption

TABLE I Adsorption of albumin on the surface of (0) poly HEMA, and copolymers HEMA-co-NaMA with (1) 1 mol %, (2) 2 mol %, (3) 3 mol % NaMA; CB ABS = light absorbance of specimens stained by Coomassie Blue R at 540 nm

Polymer	H_2O content in polymer	Albumin adsorption		
		ESCA	^{125}I	CB ABS
	(wt %)	(% atomic N)	(ng HSA cm^{-2})	540 nm
0	34.68	1.29	60	0.101
1	40.30	1.48	25	0.093
2	50.88	0.90	20	0.039
3	58.10	0.43	11	0.028

TABLE II Adhesion of macrophages (MΦ) *in vitro* and their spreading and fusion *in vivo* on the surface of (0) poly HEMA, and copolymers HEMA-co-NaMA with (1) 1 mol %, (2) 2 mol %, (3) 3 mol % NaMA.

Polymer	MΦ Adhesion serum free (%)	MΦ Spreading (μm^2)	Fusion index
0	18.40	419.00 ± 191.25	0.51 ± 0.21
1	19.12	270.00 ± 85.00[a]	0.32 ± 0.15[a]
2	10.56[a, b]	223.50 ± 96.00[a, b]	0.08 ± 0.22[a]
3	8.62[a]	200.50 ± 117.50[a]	no fusion

[a] Statistically significant difference from poly HEMA.
[b] Statistically significant difference between HEMA with 1 mol % NaMA and HEMA with 2 mol % NaMA at 0.05 significance level.

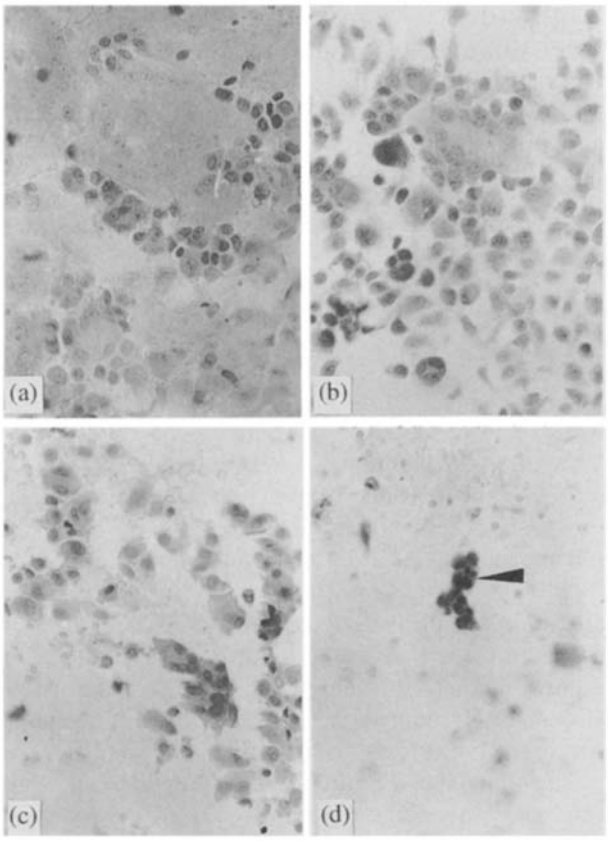

Figure 1 Cells colonizing the surface of (a) implanted poly HEMA, and copolymers (b) HEMA-co-1 mol % NaMA, (c) HEMA-co-2 mol % NaMA, and (d) HEMA-co-3 mol % NaMA; (e) foreign-body giant multinucleate cell; arrowhead, cluster of poorly spread macrophages × 260, haematoxylin–eosin.

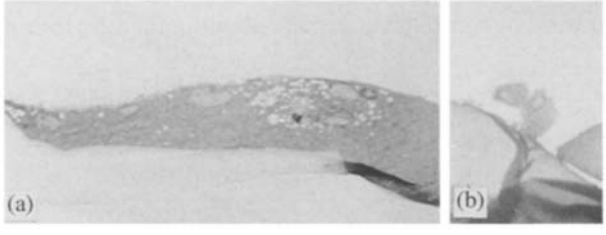

Figure 2 (a) Foreign-body giant multinucleate cell on the surface of poly HEMA, and (b) poorly spread macrophage on the surface of copolymer HEMA-co-3 mol % NaMA × 640, Toluidine Blue.

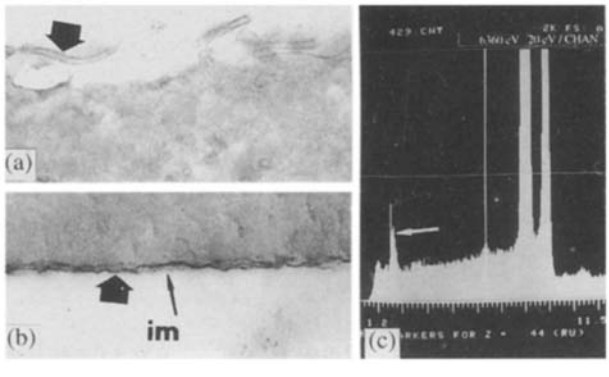

Figure 3 (a) Apical and (b) basal part of foreign-body giant multinucleate on the surface of the poly HEMA implant (im). The Ruthenium Red positivity on the cell surface (arrow), protein deposit in the cell–implant interface (small arrow) × 7000 (c) Characteristic peak (white arrow) of Ruthenium atoms on the cell surface.

and macrophage adhesion, spreading, and fusion on the polymer surface.

An increase in the concentration of anionic groups in the tested polymers decreases the adsorption of HSA on to their surface. Poly HEMA as an electroneutral polymer, binds HSA predominantly by hydrophobic interaction [8]. The copolymers with NaMA are considerably more hydrophilic (Table I) and moreover, at physiological pH, both the copolymers and HSA are negatively charged. It is known that equal signs of the surface and protein charges do not imply an absence of protein adsorption, especially at the physiological salt concentration where gross electrostatic repulsion may not be dominant. Nevertheless, as we have observed, the weakened hydrophobic interaction and the equal charge signs lead to a lower adsorption of HSA on to these polymers. It should be mentioned that adsorption of HSA on to these polymers is not strictly irreversible and slow desorption of HSA continues even after 60 min elution with PBS. While this and the data shown in Table I do not represent an irreversible component of adsorption, they do demonstrate the generally described trend. Analogically, this trend may be followed by other blood plasma proteins which are above their isoelectrical points at physiological pH.

The increased electronegativity of the implant surface decreased the *in vivo* spreading of macrophages and their subsequent fusion in foreign-body giant multinucleate cells, which corresponds to our previous observations [10, 11]. The data presented demonstrate that macrophages are able to recognize very subtle differences in the polymer surface chemical structure. The mechanism is not yet quite clear, but an intermediary role of the adsorbed proteins may be important. It is known that surface properties of implants influence conformation and activity of interacting proteins [12]. An inhibitory effect of carboxylate groups on complement activation has also been observed [13], which is very important for macrophage biology. A similar effect of anionic groups on blood platelets adhesion and thrombogenesis has been noticed, although it cannot be generalized [14–16]. Our observation of Ruthenium Red positivity on the macrophage surface corresponds to the presence of anionic sialic acids on the surface of these cells [17], which may lead to an electrostatic repulsion between a macrophage membrane and negatively charged implant surfaces.

Changes in the cell shape affect the control of gene expression [18]. Probably, fusion of the macrophages into giant foreign-body multinucleate cells is related to their spreading on the polymer surface.

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