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How do the full-generation poly(amido)amine (PAMAM) dendrimers activate blood platelets? Platelet membrane zeta potential and other membrane-associated phenomena

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ABSTRACT

We explored the hypothesis that zeta potential altered by polycations affects blood platelet activation and reactivity, the phenomena associated with membrane lipid fluidity and platelet mitochondrial bioenergetics.

PAMAM dendrimers generation- and dose-dependently enhanced zeta potential of platelets (from −10.7 mV to −4.3 mV). Increased expressions of activation markers, P-selectin and the active complex $\alpha_{IIb}\beta_3$, as well as significantly enhanced fibrinogen binding occurred upon the *in vitro* incubation of blood platelets in the presence of PAMAMs G3 and G4 (resp. 62.1% and 69.4% *vs*. 1.4% and 2.7% in control for P-selectin, $P < 0.0001$). PAMAM dendrimers increased fluidity of platelet membrane lipid bilayer, while they did not affect platelet mitochondria respiration. Increased platelet activation and their responses to agonists *in vitro* were statistically associated with the revealed alterations in zeta potential.

Our results support the hypothesis that polycation-mediated "neutralized" zeta potential may underlie the activating effects of PAMAMs on blood platelets.

1. Introduction

Poly(amido) amine (PAMAM) dendrimers are spherical, hyperbranched nanomolecules (Tomalia et al., 1984), with empty interior cavity and numerous surface moieties, what makes them convenient carriers of drugs, prodrugs or genes silencers, entrapped into dendrimers or attached to their surface (Baker, 2009). The main feature underlying the PAMAM dendrimers' biological action is their electric charge (cationic or anionic for full or half generations, respectively), derived from the surface chemical groups, like *e.g.* the primary amino groups in the full-generation PAMAM dendrimers.

Electrostatic interactions between charged moieties of dendrimers and other molecules play a pivotal role in the shaping of the pharmaceutical appearance of PAMAMs, including the encapsulation of some drugs (Cheng et al., 2008), the solubilisation of hydrophobic drugs (Huang et al., 2011) or the ability of scavenging of some toxic ions, like Cd^{2+} (Shcharbin et al., 2007). Similarly, scavenging of exceeded glucose in diabetic blood plasma has been suggested to be dependent on direct interactions between amine-terminated PAMAM G4 dendrimers and electrophilic glucose molecules (Labieniec et al., 2008; Labieniec and

Abbreviations: 5-DOXYL-Ste, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy, free radical; 12-DOXYL-Ste, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy, free radical; AA, arachidonic acid; ANOVA, analysis of variance; ATP, adenosine triphosphate; col, collagen; Da, Daltons; ESR, electron spin resonance; ETS, electron transfer system; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; Fg, fibrinogen; FITC, fluorescein isothiocyanate; G, gauss; GHz, gigahertz; HRR, high resolution respirometry; IgG_1, immunoglobulin G_1; kHz, kilohertz; L/E, quotient of LEAK respiration to the maximal FCCP-stimulated respiration; LEAK CR, LEAK control ratio; mT, millitesla; LQ, lower quartile; Me, median; MFI, mean fluorescence intensity; MW, molecular weight; mV, milivolts; PAMAM G2, poly(amido)amine dendrimers generation 2; PAMAM G3, poly(amido)amine dendrimers generation 3; PAMAM G4, poly(amido)amine dendrimers generation 4; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll; PLL, poly-L-lysine hydrobromide; PX, polymyxin; R/E, rot, rotenone; RGDS, Arg-Gly-Asp-Ser peptide; Rs, Spearman correlation coefficient; ROX, non-mitochondrial oxygen consumption; RT, room temperature; SEM, standard error of the mean; UQ, upper quartile; V, volts; UCR, uncoupling control ratio.

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Watala, 2010; Karolczak et al., 2012). Thus, electrostatic interactions appear as a key step on the way to reveal PAMAMs' biological activities, regardless of whether PAMAM dendrimers are used as carriers or they act as the compounds biologically active *per se*.

On the other side, the electric charge of PAMAM dendrimers may also be considered as one of the main causes of their toxicity and detrimental effects, like the dose- and generation-dependent destabilization of erythrocyte membranes (Domanski et al., 2004; Jain et al., 2010; Wang et al., 2010), followed by enhanced haemolysis or activated blood coagulation (Domanski et al., 2004; Greish et al., 2012).

Since blood coagulation is directly dependent on electrostatic interactions between haemostatic factors, the formation of blood clot seems to be highly prone to the influence of PAMAM dendrimers. The crucial significance of surface charge in blood coagulation (Chargaff et al., 1941; Wolfrom and Rice, 1947) and the role of electrostatic interactions in stabilizing of numerous haemostatic factors (Karshikov et al., 1992) have been recognized previously.

Blood platelets, the main cellular particles driving haemostasis, bear on theirs surface a zeta potential equal to −11.31 mV (Coller, 1983). Under physiological conditions this negative charge, constituted by non-protein and protein carboxyls and by phosphate residues (Seaman, 1975), largely underlies the generation of repulsive forces in a bloodstream, thus keeping platelets as non-clustered particles (Coller, 1983). We have to remain aware however, that it is the negative charge of membrane phospholipids, together with platelets' ability of temporal loss of membrane phospholipid distribution asymmetry, which make the anionic charge of platelet surface membranes to matter that much (Dachary-Prigent et al., 1995; Pasquet et al., 1997).

Thus, cationic PAMAM dendrimers have at least a few potential targets in platelet membranes, which determine their mutual electrostatic interactions. It is likely that the vanishing of an overall blood platelet anionic charge through their interacting with cationic PAMAM dendrimers has a considerable impact on platelet membrane lipid bilayer fluidity or the binding of crucial physiological ligands to platelet membrane receptors, and thus may result in altered haemostatic properties of platelets. On the other hand, cationic PAMAM dendrimers may impair the functioning of platelet mitochondria, and hence, by impacting platelet bioenergetics, they are likely to considerably up-regulate platelet metabolism, activation and aggregation (Barile et al., 2012; Tomasiak et al., 2004). Indeed, the recent *in vitro* studies have shown size- (generation-) and surface charge-dependence of PAMAM-induced platelet aggregation (Dobrovolskaia et al., 2012; Jones et al., 2012), especially by positively charged PAMAM dendrimers (Jones et al., 2012). Despite numerous suggestions on the putative significance of electrostatic charges in the interactions of PAMAM dendrimers with blood cells, there is a lack of a direct evidence showing the importance of electrostatic interactions between dendrimeric polycations and platelets.

Zeta potential measurement is a method of choice when investigating an overall cellular electrical charge, including blood platelets (Friedhoff and Sonenberg, 1983; Ribeiro et al., 2012; Tatsumi et al., 1992). Thus, we have performed the measurements of zeta potential in the intact isolated blood platelets incubated with PAMAM dendrimers G2, G3 and G4. We have expected that reduced zeta potential values of resting platelets directly correspond to the increased expressions of two major hallmarks of blood platelet activation: the release of P-selectin of intraplatelet α-granules and the activation of fibrinogen receptor, the GPIIb/IIIa complex ($\alpha_{IIb}\beta_3$). Thus, the leading hypothesis assumed that the vanishing of repulsive forces between blood platelets due to their interactions with PAMAM cationic dendrimers leads to cell activation, marked as accelerated release reaction, facilitated inside–out signaling, enhanced fibrinogen binding, reduced platelet membrane fluidity and increased mitochondrial respiration.

2. Materials and methods

2.1. Chemicals

Commercially available methanol solutions of PAMAM dendrimers: PAMAM dendrimer, ethylenediamine core, generation 4.0 (PAMAM G4, $[NH_2(CH_2)_4NH_2]$:($G = 4$); dendri PAMAM$(NH_2)_{64}$; MW 14215 Da), generation 3.0 (PAMAM G3, $[NH_2(CH_2)_4NH_2]$:($G = 3$); dendri PAMAM$(NH_2)_{32}$; MW 6909 Da) and PAMAM dendrimer, ethylenediamine core, generation 2.0 (PAMAM G2, $[NH_2(CH_2)_4NH_2]$:($G = 2$); dendri PAMAM$(NH_2)_{16}$; MW 3256 Da) (10%, 20% and 20% w/w, respectively), poly(L-lysine) (PLL, poly-L-lysine hydrobromide, mol wt. 15,000–30,000 Da), polymyxin (PX), rotenone (rot), oligomycin, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), antimycin A and two spin labels: 5-DOXYL-Ste (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy, free radical, 5-doxylstearate, 5-SASL) and 12-DOXYL-Ste (2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy, free radical) were purchased from Sigma–Aldrich (Frankfurt, Germany). Reagents for flow cytometry studies (anti-human mAb CD61/PerCp, CD62P/PE, PAC-1/FITC, CellFix and Vacutainer™ containing 0.105 M buffered sodium citrate) were from Becton Dickinson (San Diego, CA, USA). Oregon Green 488—conjugated human fibrinogen was from Molecular Probes (Eugene, OR, USA). Integrilin™ was commercially available from Merck & Co., Inc. (Whitehouse Station, NJ, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Polish Chemical Reagents (POCh, Gliwice, Poland), unless otherwise stated. The water used for the solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, Thermo Scientific, Waltham, MA, USA).

2.2. Blood collection

Human blood was collected from healthy donors (altogether, 34 individuals: 15 women and 19 men; mean age 30.6 ± 5.7 years). This part of the study was performed under the guidelines of the Helsinki Declaration for human research and was approved by the local Committee on the Ethics of Research in Human Experimentation at Medical University of Lodz. Written informed consent, including detailed information regarding the study objectives, design, risks and benefits, was obtained from each individual before blood withdrawal. None of the donors had taken aspirin or other drugs affecting platelet function for at least 10 days prior to blood collection or had a history suggestive of underlying haemostatic disorders. Blood was withdrawn on 105 mol/l sodium citrate (citrate: blood volume ratio 1:9), with a special caution to avoid undesirable activation of circulating platelets.

2.3. Isolation of blood platelets and preparation of working solutions of polycations

Human platelets were isolated from whole blood by the method of differential centrifugation (McNicol, 1996). The isolated platelets were resuspended in the PBS buffer (150 mmol/l NaCl, 1.9 mmol/l NaH_2PO_4, 8.1 mmol/l Na_2HPO_4, pH 7.4) in the case of samples for measurements of Zeta potential or in a Tyrode's buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 3 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l $NaHCO_3$, 10 mmol/l glucose, pH 7.4) in the case of the samples for flow cytometry and ESR measurements, and their