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How do the full-generation poly(amido)amine (PAMAM) dendrimers activate blood platelets? Activation of circulating platelets and formation of "fibrinogen aggregates" in the presence of polycations

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ABSTRACT

Direct use of poly(amido)amine (PAMAM) dendrimers as drugs may be limited, due to uncertain (cyto) toxicity. Peripheral blood components, which constitute the first line of a contact with administered pharmaceuticals, may become vastly affected by PAMAM dendrimers. The aim of this study was to explore how PAMAMs' polycationicity might affect blood platelet activation and reactivity, and thus trigger various haemostatic events.

We monitored blood platelet reactivity in rats with experimental diabetes upon a long-term administration of the unmodified PAMAM dendrimers. In parallel, the effects on blood flow in a systemic circulation was recorded intravitally in mice administered with PAMAM G2, G3 or G4. Compounding was the *in vitro* approach to monitor the impact of PAMAM dendrimers on blood platelet activation and reactivity and on selected haemostatic and protein conformation parameters. We demonstrated the activating effects of polycations on blood platelets. Some diversity of the revealed outcomes considerably depended on the used approach and the particular technique employed to monitor blood platelet function. We discovered undesirable impact of plain PAMAM dendrimers on primary haemostasis and their prothrombotic influence. We emphasize the need of a more profound verifying of all the promising findings collected for PAMAMs with the use of well-designed *in vivo* preclinical studies.

1. Introduction

Poly(amidoamine) (PAMAM) (Starburst™) dendrimers are circular or spherical synthetic polymers characterized by unique, well-defined structural features. Numerous free surface amino groups covering dendrimers (the number of terminal amine groups grows directly to PAMAM dendrimer generation) are more or less susceptible to react with various nucleophilic molecules and are potentially accessible for attachment of required compounds, e.g. gene silencers (Pan et al., 2009). Dendrimers are suggested as "Trojan horses" for other molecules (Medina and El Sayed, 2009), but may also solely serve as pharmaceuticals (Xue et al., 2013). However, the exact PAMAMs' pharmacological activity has not been strictly established. Despite very interesting features of dendrimers revealed in the *in vitro* model studies (Klajnert et al., 2006, 2007; Labieniec and Watala, 2010), their behaviour in living organism has only very occasionally been addressed (Greish et al., 2012; Hong et al., 2004, 2009; Karolczak et al., 2012; Labieniec et al., 2008; Yang et al., 2012). Thus, the direct use of PAMAMs,

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; AGEs, advanced glycation end-products; ALT, alanine aminotransferase; ANOVA, analysis of variance; AOPP, plasma advanced oxidation protein products; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; AUC, area under the curve; b.w., body weight; Da, daltons; EDTA, ethylenediaminetetraacetic acid; EMG, exponentially-modified Gaussian distribution; Fg, fibrinogen; FITC, fluorescein isothiocyanate; HbA_{1c}, glycated haemoglobin; IgG, immunoglobulin G_1; IgM, immunoglobulin M; LDF, lasser Doppler flowmetry; LDH, lactate dehydrogenase; LDU, arbitrary laser Doppler units; LQ, lower quartile; Me, median; moAbs, monoclonal antibodies; PAMAM G2–G7, poly(amido)amine dendrimers generation 2–7; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll; PLL, poly-L-lysine hydrobromide; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PT, prothrombin time; PX, polymyxin; RT, room temperature; SD, standard deviation; SEM, standard error of the mean; STZ, streptozotocin; T_r, time needed for the occlusion/aggregation, measured between 10% and 90% of maximal response; Trp, tryptophan; TT, thrombin time; UQ, upper quartile; V_{0r}, the maximal rate of the blood flow decrease averaged through T_r; V_{avgr}, the mean rate of the blood flow decrease averaged through T_r.

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either as drugs or drug carriers, is still seriously limited mainly due to largely uncertain side effects and their (cyto)toxicity. Peripheral blood components may become vastly affected by PAMAM dendrimers, simply because they most often constitute the first line of a contact with any ingested pharmaceuticals. Dendrimers are very often administered intravenously or intraperitoneally. Hence, they regularly get into bloodstream and encounter blood cells and plasma components. This naturally implies interactions of the nanoparticles with plasma proteins, blood cells and vascular endothelial cells, even before PAMAMs achieve their supposed target tissues. Interactions with blood cells are of particular interest also in the context of some earlier reports on the experimental use of PAMAM dendrimers as anti-diabetic nanopharmaceuticals, and more even so, considering the biotoxicity of these nanomolecules despite the potential benefits concerning the hypoglycaemizing effects revealed in animals with streptozotocin-induced diabetes (Labieniec et al., 2008; Labieniec-Watala et al., 2013, 2014). The polycationicity of PAMAM dendrimers enables them to directly interact with various anionic targets on cell surface, including membrane receptors located on blood platelets, which are the key molecules in the phenomena of platelet activation and haemostatic events. Consequences of such hypothetical interactions between PAMAM dendrimers and blood components participating in haemostasis still remain largely unexplored.

In the present study we used two experimental approaches to investigate the overall impact of full generation PAMAM dendrimers on blood primary haemostasis and blood platelets in particular. The *in vivo* study involved a long-term administration of the unmodified (plain) PAMAM polymers to rats with experimental diabetes and the *ex vivo* monitoring of the effects of the dendrimers on blood platelet reactivity. In parallel, blood flow in a systemic circulation was recorded intravitally in mice administered with PAMAM dendrimers. In the second approach the impact of various polycationic compounds, including PAMAM dendrimers and two reference polyamines, on blood platelet reactivity and on a few other haemostatic and protein conformation parameters was analysed upon the *in vitro* incubation of whole blood or isolated blood platelets, plasma or plasma proteins with the polycations. In general, we demonstrated the activating effects of polycations on blood platelets, however, some diversity of the revealed outcomes depended on the used approach and on the particular technique employed to monitor blood platelet function. We employed several compounding and supplementary laboratory techniques and assays in order to resolve the possible sources of these diverse outcomes.

2. Materials and methods

2.1. Chemicals

Insoluble equine tendon collagen type I and arachidonic acid (AA) was from Chrono-Log Corp. (Havertown, PA, USA). Fluorolabelled monoclonal antibodies (moAbs): anti-CD61/PerCP, anti-CD62/PE, PAC-1/FITC, isotype controls IgG/PE and IgM/FITC and other materials for flow cytometry were from Becton Dickinson (San Diego, CA, USA). Calcein AM was from Invitrogen (Eugene, OR, USA). Reagents for gel electrophoresis were from Sigma–Aldrich (St. Louis, MO, USA) and Bio-Rad (Hercules, CA, USA). CytoTox-ONE™ Homogeneous Membrane Integrity Assay was from Promega (Madison, WI, USA). Ketamine and sedazin for animal anaesthesia were purchased from Biowet-Pulawy (Poland). Anticoagulant-hirudin (Refludan®) was from Aventis Pharma Deutschland GmbH (Bad Soden a. Ts., Germany). Tris-glycine native buffer, pH 8.3 was from Bio-Rad Laboratories (Hercules, CA, USA). Commercially available methanol solutions of PAMAM dendrimers: PAMAM dendrimer, ethylenediamine core, generation 4.0 (PAMAM G4, $[NH_2(CH_2)_4NH_2]$:(G4); dendri PAMAM$(NH_2)_{64}$), PAMAM dendrimer, ethylenediamine core, generation 3.0 (PAMAM G3, $[NH_2(CH_2)_4NH_2]$:(G3); dendri PAMAM$(NH_2)_{32}$) and PAMAM dendrimer, ethylenediamine core, generation 2.0 (PAMAM G2, $[NH_2(CH_2)_4NH_2]$:(G2); dendri PAMAM$(NH_2)_{16}$) (10% for G4 and 20% w/w for G2, G3), poly(L-lysine) (PLL, poly-L-lysine hydrobromide, mol.wt. 15,000–30,000 Da), polymyxin (PX) and fibrinogen (Fg) from human plasma were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were from Polish Chemical Reagents, unless otherwise stated. Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, IA, USA).

Stock solutions of PLL and PX were prepared in distilled water on the day of use and further diluted in phosphate-buffered saline (PBS: 150 mmol/l NaCl, 1.9 mmol/l NaH_2PO_4, 8.1 mmol/l Na_2HPO_4, pH 7.4), as required. The methanol stock solutions of PAMAM dendrimers were diluted in PBS prior to their addition to blood samples. All diluted compounds were prepared freshly and used on the same day. Accordingly, the control samples for PLL or PX contained PBS, while the control samples for PAMAM dendrimers contained methanol diluted in PBS. Our choice of PAMAM dendrimers concentrations used in the present study has been governed by the recognition of the balance between toxicity and non-toxic biological effects reported earlier (Labieniec-Watala et al., 2013). PLL and PX were used as the reference polyamine compounds.

2.2. Administration of PAMAM dendrimers to non-diabetic and STZ-diabetic rats and blood collection

All animal experiments were done in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (US National Institute of Health No. 85-23, revised 1985) and with the guidelines of the European Community for the Use of Experimental Animals (L358-86/609/EEC) and the Guiding Principles in the Use of Animals in Toxicology (1989), after earlier acceptance of the experiment layout by the local Committee on Standardization and Ethics for Experiments with Animals.

One hundred male Wistar rats (at the beginning of the experiment weighing 250–300 g) were used in the experiment. Rats were bred in the groups counting three animals *per* one cage, with a free access to standard laboratory chow Murigran (Motycz, Poland,) and tap water. The 12-h light:12-h darkness cycle was kept in a cultured room.

Based on our earlier study (Labieniec et al., 2008), we adopted the follow-up time of 60 days after recruitment and the accrual time of 5 days, during which we recruited animals to the experiment.

We randomly selected 50 rats (the number of rats in accordance with the results of earlier minimum sample size estimation) for the induction of experimental diabetes. Diabetes was induced by the intraperitoneal injection of streptozotocin (STZ) dissolved in 0.1 mol/l citrate buffer (pH 4.5) and given at a dose of 60 mg/kg body weight (b.w.). Diagnosis of diabetes was made on the basis of the monitoring of blood glucose concentration (08:00–09:00 AM). We included to the study only the rats showing blood glucose concentrations higher than 16.7 mmol/l. Each STZ-injected rat showing hyperglycaemia lower than 16.7 mmol/l at 72 h after STZ injection, was considered as “non-diabetic” and excluded from the further study.

The part of the experiment involving the administration of PAMAM G4 to animals started after 7 days upon the induction of a laboratory confirmed diabetes. The groups of diabetic and non-diabetic animals were further divided into two subgroups each: the first receiving a vehicle (methanol dissolved in PBS) and the second