Haemolytic activity of polyamidoamine dendrimers and the protective role of human serum albumin

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We have examined the haemolytic activity of polyamidoamine dendrimers. Haemolysis increased in a generation- and concentration-dependent manner. Moreover, the longer the incubation time, the greater the amount of haemoglobin released. When human serum albumin (HSA) was present in the incubation buffer, the extent of haemolysis decreased. This protective effect may be due to the high affinity of dendrimers for proteins: dendrimers that interact with HSA are unable to disrupt the membrane to the same extent as free dendrimers. The presence of HSA makes the buffer more relevant to physiological conditions. The results of this study suggest that the actual haemotoxicity of dendrimers in vivo is lower than is observed in vitro.

Keywords: dendrimer; PAMAM; HSA; haemolysis; toxicity

1. Introduction

Polyamidoamine (PAMAM) dendrimers were one of the earliest types to be synthesized (Tomalia et al. 1985). Like all dendrimers, they are characterized by a globular shape, many surface end groups and empty internal cavities. This unique structure has made them promising materials for various applications, including biomedical ones (Lee et al. 2005; Svenson & Tomalia 2005). Although the use of dendrimers as therapeutic agents is a rapidly growing field, there are still concerns about their toxicity and their effects on biological systems. One of the most convenient ways to administer drugs based on dendrimers is by intravenous injection (Okuda et al. 2006). However, if that is done, blood constituents are likely to be the first and unwanted targets of their action; in particular, haemolysis can occur. Haemolysis is the release of haemoglobin from damaged erythrocytes (also known as red blood cells). It is well known that haemolysis can be caused by various factors, e.g. hypotonic solutions and toxic compounds. It is always a consequence of serious damage to the membrane lipid bilayer and the loss of its integrity. The surface of the red blood cells is negatively charged under physiological conditions. Therefore, dendrimers and other macromolecules with exposed cationic surface groups have haemotoxic
effects, due to disruption of the cell membrane through initial adhesion to the cell surface by electrostatic attraction, probably followed by the formation of holes (Hong et al. 2004).

In this study, we investigated the haemolytic activity of PAMAM dendrimers (generations 4–6). This is a continuation of our earlier work, in which lower generations were investigated (Domanski et al. 2004). Since dendrimers have high affinities for proteins (Klajnert et al. 2003), we checked whether the presence of human serum albumin (HSA) in the incubation buffer protected erythrocyte membranes against disruption.

2. Material and methods

PAMAM dendrimers (generations 4–6) and HSA were purchased from Sigma-Aldrich (Germany). Blood from healthy donors obtained from the Central Blood Bank (Lodz) was anticoagulated with 3 per cent sodium citrate. Erythrocytes were separated from the plasma and leucocytes by centrifugation (1000 g, 5 min) at 4°C and washed three times with phosphate-buffered saline (PBS: 150 mM NaCl, 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4). For microscopy, erythrocytes at a haematocrit of 2 per cent were suspended in solutions containing 4.5 mg ml⁻¹ PAMAM G5 dendrimers and incubated at 37°C for 2 h. Ten-fold diluted samples were then viewed under a Nicon Eclipse 500 optical microscope at a magnification of 400 ×.

To measure the haemolysis caused by the dendrimers, erythrocytes at a haematocrit of 2 per cent (the proportion of the blood volume occupied by red blood cells) were suspended in dendrimer solutions at concentrations ranging from 0.5 to 6 mg ml⁻¹ and incubated at 37°C for 2, 6, 14 and 24 h. Stock dendrimer solutions were dissolved in PBS. The erythrocyte suspensions were centrifuged (1000 g, 5 min) and the percentage haemolysis was determined on the basis of haemoglobin released into the supernatants, measured spectrophotometrically at 540 nm. For reference (100% haemolysis), red blood cells were treated with distilled water.

To study the effect of HSA, 2 mg ml⁻¹ of HSA was added to 4.5 mg ml⁻¹ of PAMAM G5 dendrimer solution. After 5 min, red blood cells were added to 2 per cent haematocrit. The time to equilibrate the system (5 min) was chosen on the basis of previous observations of changes in the intrinsic fluorescent properties of HSA, which stabilized after 2–3 min (Klajnert et al. 2003). The erythrocytes were then incubated for 2, 6 and 24 h, and haemolysis was determined as described above.

3. Results

The influence of dendrimers on red blood cell morphology was checked by optical microscopy. Most of the control cells were discocytes (figure 1a). They had a typical biconcave disc shape, which is normal under physiological conditions. In the presence of 1.5 mg ml⁻¹ of PAMAM G5, echinocytic transformation occurred (figure 1b). Echinocytes are morphologically altered red blood cells that appear to have numerous uniform spicules throughout the cell membrane.
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Figure 1. Photographs of control erythrocytes (haematocrit 0.2%) (a); erythrocytes (haematocrit 0.2%) after 2h incubation at 37°C in 1.5 mg ml\(^{-1}\) of PAMAM G5 (b); erythrocytes (haematocrit 0.2%) after 2h incubation at 37°C in 4.5 mg ml\(^{-1}\) of PAMAM G5 (c). Magnification 400\(\times\). Arrows indicate an echinocyte (b) and an echinocytic transformation that leads to cell disruption (c).

Obviously, a certain number of erythrocytes underwent lysis, so the number of red blood cells was lower. When the dendrimer concentration was higher (4.5 mg ml\(^{-1}\)), the effect was more pronounced and fewer erythrocytes were visible. This means that most of them had been destroyed. Only a few retained their physiologically normal shape. Cells undergoing lysis were also visible (figure 1c).
Figure 2. Haemolysis induced by PAMAM dendrimer generation 4 (a), generation 5 (b) and generation 6 (c). The statistical significance of the results, expressed as mean ± s.d. was determined by comparison with control values using Student’s t-test. ∗p < 0.05, ∗∗p < 0.005, ∗∗∗p < 0.0005, n = 6.

The changes in morphology were accompanied by dendrimer-induced haemolysis. The process was generation- and concentration-dependent (figure 2). The fourth generation was the least haemolytic and the sixth generation was the most haemolytic. This indicates that the number of cationic surface groups determines the activity. The dendrimer concentrations were selected to achieve approximately 50 per cent haemolysis. In the case of PAMAM G4, the effect was less pronounced even though the concentration applied was higher than for PAMAM G5 and PAMAM G6. The longer the erythrocytes were incubated with dendrimers, the more haemolysis was observed; but interestingly, no significant difference was observed between 14 and 24 h incubation in most cases. Each dendrimer concentration corresponded to a certain degree of haemolysis-inducing damage. When this level was reached, prolonging the incubation did not cause...
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Figure 3. Haemolysis of red blood cells in the presence of 4.5 mg ml\(^{-1}\) of PAMAM G5 dendrimers and 2 mg ml\(^{-1}\) of HSA. The results are expressed as mean ± s.d., \(n = 6\). Open square, control; filled square, PAMAM G5; striped square, PAMAM G5 + HSA.

...statistically significant changes. For PAMAM G5 and PAMAM G6, 4.5 mg ml\(^{-1}\) turned out to be the critical concentration and caused much more haemolysis than 3 mg ml\(^{-1}\) (especially after 2 h incubation).

Adding HSA to the system significantly reduced the amount of haemolysis caused by PAMAM G5 dendrimers (figure 3). The concentration of albumin (2 mg ml\(^{-1}\)) was chosen to achieve the same ratio of haematocrit to albumin concentration as under physiological conditions (Ben-Ami et al. 2003).

4. Discussion

PAMAM dendrimers are polymers with a cationic surface consisting of amino groups. The number of amino groups depends on the generation of dendrimers. For PAMAM G4, PAMAM G5 and PAMAM G6, the numbers of functional groups are 64, 128 and 256, respectively. The charge is crucial for toxicity, including haemotoxicity (Malik et al. 2000). Red blood cell lysis is a simple and widely used method for studying membrane alterations. It gives a quantitative measure of a haemoglobin release. The data obtained in such assays also give a qualitative indication of the damage that dendrimers may potentially cause when administered intravenously. Although the mechanisms of interaction between cell membranes and dendrimers are not yet completely understood, it is safe to state that dendrimers can interact with both the lipid bilayer and certain membrane proteins. The best-established hypothesis says that dendrimers change the membrane curvature (Zhang & Smith 2000; Ottaviani et al. 2002) and create holes (Hong et al. 2004). Dendrimers interact with lipid phosphate headgroups, causing partial dendrimer incorporation into the lipid bilayer and also pulling out the outer leaflet (figure 4). The higher the generation, the more groups are present on the surface; this may explain why haemolysis is generation-dependent. Earlier studies showed that haemolysis is usually preceded by echinocytic transformation (Malik et al. 2000; Domanski et al. 2004). The red blood cells display a characteristic, irregular contour owing to folding of the periphery. Drephanocyte-like forms (rigid cells characterized by a sickle shape) were recorded by Domanski et al. (2004) at the highest dendrimer concentrations tested. Malik et al. (2000)
also observed spheroechinocytes (round, rigid, undeformable cells), although echinocytic transformation predominated. Our present results are consistent with those observations.

Not only do dendrimers interact with membrane proteins, their generally high affinity for proteins and peptides has been described in several publications (Ottaviani et al. 2004; Jokiel et al. 2006; Klajnert et al. 2006). The first studies on interactions between dendrimers and proteins focused on serum albumins. As major soluble protein constituents of the circulation, serum albumins have many physiological functions and play a key role in the transport of many endogenous and exogenous ligands. Serum albumins are the principal carriers of fatty acids, which are otherwise insoluble in the blood and have a high affinity for haematin, bilirubin and small negatively charged hydrophobic molecules. It has been proposed that PAMAM dendrimers create a layer on the surface of the serum albumin molecule (Klajnert et al. 2003). Electrostatic interactions are the driving forces in this process. At physiological pH, HSA has a negative charge, favouring interactions with cations. It is worth mentioning that these interactions do not perturb protein function, e.g. HSA is still able to bind the fluorescent dye 1-anilinonaphthalene-8-sulfonic acid (ANS) in the presence of dendrimers (Shcharbin et al. 2005). This is very important, since binding to serum albumin

**Figure 4.** Models of interactions between PAMAM G4 dendrimers and a lipid bilayer (A and C) (Ottaviani et al. 2002; Hong et al. 2004), a membrane protein (B) and HSA (D). Adapted from Shcharbin et al. (2007), five molecules of PAMAM G4 interact with one molecule of HSA.
is a critical determinant of biodistribution and pharmacokinetics for many drugs. To sum up, dendrimers cause no undesirable effects by virtue of their high affinity for HSA. Moreover, serum albumin in the incubation medium significantly diminished the membrane perturbations caused by dendrimers. Haemotoxicity is most commonly assessed in buffers (Fischer et al. 2003; Chen et al. 2004; Agashe et al. 2006; Bermejo et al. 2007). Under physiological conditions, red blood cells are suspended in the plasma, which contains plasma proteins, among which serum albumin is the most abundant. The partial suppression of haemolysis in the presence of the protein is important for assessing the overall toxicity of dendrimers in model systems. It means that dendrimers that interact with proteins are significantly less harmful to red blood cells (figure 4).

References


