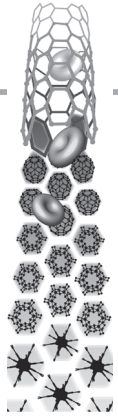

Dendrimer-induced leukocyte procoagulant activity depends on particle size and surface charge

Aims: Thrombogenicity associated with the induction of leukocyte procoagulant activity (PCA) is a common complication in sepsis and cancer. Since nanoparticles are increasingly used for drug delivery, their interaction with coagulation systems is an important part of the safety assessment. The purpose of this study was to investigate the effects of nanoparticle physicochemical properties on leukocyte PCA, and to get insight into the mechanism of PCA induction. **Materials & Methods:** A total of 12 formulations of polyamidoamine (PAMAM) dendrimers, varying in size and surface charge, were studied *in vitro* using recalcification time assay. **Results:** Irrespective of their size, anionic and neutral dendrimers did not induce leukocyte PCA *in vitro*. Cationic particles induced PCA in a size- and charge-dependent manner. The mechanism of PCA induction was similar to that of doxorubicin. Cationic dendrimers were also found to exacerbate endotoxin-induced PCA. **Conclusion:** PAMAM dendrimer-induced leukocyte PCA depends on particle size, charge and density of surface groups.

KEYWORDS: coagulopathy ■ disseminated intravascular coagulation ■ leukocytes ■ PAMAM dendrimers ■ procoagulant activity ■ thrombosis

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Leukocyte procoagulant activity (PCA) is accepted as an important component in the onset of disseminated intravascular coagulation (DIC). DIC is marked by formation of blood clots in blood vessels throughout the body and is common in acute promyelocytic leukemia (APL) and other forms of cancer [1–5] as well as sepsis [6–9]. DIC in cancer patients is often observed after initiation of therapy with cytotoxic oncology drugs that act by altering DNA replication (e.g., doxorubicin, daunorubicin and vincristin) [3,10]. Cytotoxic oncology drugs acting by other mechanisms (e.g., methotrexate and paclitaxel) do not induce DIC [11,12].

Cytotoxic drugs known to cause DIC *in vivo* have previously been shown to induce leukocyte PCA *in vitro* [3,13–20]. Endotoxin has also been linked to both DIC and PCA [6–9,21,22]. Given this established relationship between PCA and DIC, tendency to induce PCA *in vitro* can be used to screen for potential DIC.

Our laboratory, NCI's Nanotechnology Characterization Lab (NCL), has characterized over 220 different nanomaterial-based drug formulations, including dendrimers, nanoemulsions, nanoliposomes, quantum dots, fullerens, and various metal and metal oxide nanoparticles. Recent observations of DIC-like reactions to nanomaterials have motivated us to examine the tendency of various materials to induce PCA and to attempt to relate PCA induction to nanomaterial physicochemical properties, such as size and charge. Many cell types, including endothelial cells, leukocytes and cancer cells, can express PCA [12,17,18]. The focus of the present study was on normal and malignant leukocytes.

Dendrimers are among several types of nanomaterials that have a record of use in clinical studies [23,24]. These materials are considered attractive for drug delivery because they are monodisperse, uniform, hyperbranched, protein-like structures with well-defined synthesis [25]. In addition to these properties, polyamidoamine (PAMAM) dendrimers are also commercially available and can be synthesized in large quantities.

As dendrimers are increasingly developed for drug delivery, their immunological compatibility must be assessed in order to establish their safety for clinical use. Several studies have reported dendrimer interactions with blood components, such as erythrocytes and monocytes [26–29], although none, to our knowledge, examine the potential to induce blood coagulation through leukocyte PCA.

The purpose of this study was to determine if dendrimers induce PCA *in vitro* and to examine the relationship between any such PCA induction and dendrimer structure. To study PCA, we used two models: peripheral blood mononuclear cells (PBMCs) from healthy donor volunteers, as a model of normal leukocytes; and HL-60 cells as a model of acute promyelocytic leukemia (a common form of blood cancer). Both PBMCs

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and HL-60 cells have a long record of use in *in vitro* PCA studies [20] and estimating potential to induce DIC [13]. We tested 12 formulations of PAMAM dendrimers, selected to allow comparison of dendrimers with similar sizes but different surface charges, and similar charges but different sizes. The study included four generations (G_3, G_4, G_5 and G_6), and three surface functionalities (succinamic acid [carboxy-terminated], amine [amine-terminated], and amidoethanol [hydroxyl-terminated]) corresponding to three surface charges (anionic, cationic and neutral, respectively).

Herein we report that irrespective of particle size, anionic and neutral PAMAM dendrimers did not induce PCA in either normal or cancer cells *in vitro*. Cationic dendrimers induced PCA in cancer cells, but not normal cells, and the degree of induced PCA was proportional to the particle size and number of surface amines (surface charge). In normal leukocytes (PBMC model), the cationic dendrimer formulations enhanced endotoxin-mediated PCA. We also demonstrated that the mechanism of dendrimer-induced PCA induction is similar to that induced by DNA-intercalating cytotoxic oncology drug doxorubicin, in that it requires *de novo* protein synthesis, *de novo* RNA synthesis, and involves phospholipid and tissue factor expression on cell surfaces.

Materials & methods

Reagents

Ca^{2+}/Mg^{2+} free DPBS, cycloheximide, actinomycin D, and phospholipase C were from Sigma-Aldrich (St.Louis, MO, USA). Endotoxin standard for LAL assay, LAL-grade water and Limulus Amoebocyte Lysate were from Associates of Cape Code Inc. (East Falmouth, MA, USA). *Escherichia coli* K12 ultrapure lipopolysaccharide (LPS) was obtained from Invivogen Inc (San Diego, CA, USA). Ficoll Paque Plus was purchased from GE HealthCare (Waukesha, WI, USA). Tissue factor neutralizing antibody and isotype control antibody were acquired from American Diagnostica Inc (product #4501, Stamford, CT, USA) and Jackson ImmunoResearch Labs (West Grove, PA, USA), respectively. Doxorubicin Hydrochloride (Adriamycin-TM) was obtained through NIH pharmacy.

Dendrimers

1,4-diaminobutane core, G_3, G_4, G_5 and G_6 PAMAM dendrimers with amine, succinamic acid and amidoethanol surfaces were purchased from Dendritic Nanotechnologies Inc. (Mount Pleasant, MI, USA). Amine- and amidoethanol-terminated dendrimers were supplied as 10% (w/v) solution in methyl alcohol, and succinamic acid terminated dendrimers were supplied as 10% (w/v) solution in water. All formulations were stored at -20°C. All formulations were stored in borosilicate glass vials. On the day of experiment aliquots containing approximately 1.0 mg of amine- and amidoethanol-terminated dendrimers were removed from original storage containers after the containers were allowed to equilibrate to room temperature and the methanol was removed by entraining with nitrogen. Aliquots of succinamic acid-terminated dendrimers were lyophilized to remove storage media. After that all formulations were reconstituted in water to a final concentration of 10.0 mg/ml. The pH of the samples was approximately 7 for all samples as follows: that of all $–NH_2$ terminated samples was adjusted to 7.3 using 0.1 M HCl; the pH of hydroxy-terminated samples was 7.0, 7.1, 7.0, 6.6 for G_3, G_4, G_5, G_6 dendrimers, respectively, and did not require adjustment; the pH of carboxy-terminated dendrimers was adjusted to 7.0, 7.5, 7.5, 7.3 for G_3, G_4, G_5, G_6 dendrimers, respectively, using 0.1 M NaOH.

For physicochemical characterization particles were diluted in PBS and/or 10 mM sodium chloride to mimic physiologically relevant conditions. For cell culture experiments the samples were diluted into cell culture medium. Stocks in water were stored for no longer than 1 week at 4°C. Samples diluted to a final working concentration in either PBS, sodium chloride or culture medium were used within 2 h of preparation during which the particles were kept at room temperature. Unused portion of aliquots were discarded.

Particle characterization

LAL assay to detect & measure levels of bacterial endotoxin

Analysis was performed using PyrosKinetix instrument of Associates of Cape Code Inc. Assay range for kinetic turbidity LAL was from 0.001 to 1.0 EU/ml. Standard curves (calibration curves) were prepared by spiking known amounts of USP-certified endotoxin standard into endotoxin-free (LAL-grade) water. In addition to the standard curve, each individual run of each LAL assay included a set of quality controls: positive quality controls for each assay were prepared by spiking endotoxin standard, at the standard curve midpoint concentration, into LAL-grade water. Inhibition/enhancement controls (IEC)

were prepared by spiking the same amount of endotoxin standard used in quality controls into the nanoparticle formulation. Results from each individual assay run were not considered valid unless the precision and accuracy of the standard curve and quality control were within 25% and the inhibition/enhancement control exhibited 50–200% spike recovery. These acceptance criteria are in accordance with those mandated by the US FDA guidelines and USP standard for the LAL test [30,31]. Each nanoparticle sample and inhibition/enhancement controls was tested in duplicate and repeated three times. For each formulation at least three dilutions of the test sample were tested.

Dynamic light scattering

A Malvern Zetasizer Nano ZS instrument (Southborough, MA, USA) with back scattering detector (173°, 633 nm laser wavelength) was used for measuring the hydrodynamic size (diameter) in batch mode at 25°C in a low volume quartz cuvette (pathlength 10 mm). Dendrimer samples were prepared at a concentration of 1 mg/ml in PBS and filtered through a 0.02 µm filter. A minimum of 12 measurements per sample were made. Hydrodynamic size is reported as the intensity-weighted average (Int-Peak). Analysis of particle size after dilution into cell culture medium containing serum was not possible due to interference of protein with the instrument measuring technique.

Zeta potential

Zeta potential provides a measure of the electrostatic potential at the surface of the electrical double layer and the bulk medium, which is related to the nanoparticle surface charge. A Malvern Zetasizer Nano ZS instrument was used to measure zeta potential at 25°C. PAMAM dendrimer samples were prepared at a concentration of 10, 2 and 1 mg/ml, respectively, in 10 mM NaCl. An applied voltage of 100 V was used. Samples were loaded into prerinsed folded capillary cells and a minimum of three measurements were made per sample. Analysis of particle charge after dilution into cell culture medium containing serum was not possible due to interference of protein with the instrument measuring technique.

Leukocyte PCA

Research donor blood

Healthy volunteer blood specimens were drawn under NCI-Frederick Protocol OH99-C-N046. Blood was collected in BD vacutainer tubes containing sodium citrate as anticoagulant. Blood was kept at room temperature. Within 1 h after collection blood was span down at 2500 ×g for 10 min at room temperature and platelet free plasma was collected into fresh tubes. For experiments using a primary cell model, autologous individual plasma specimens were used. To test the effect of leukemia cells (HL-60 model) on plasma coagulation and to avoid interindividual variability, plasma specimens from at least three donors were pooled.

Leukocyte PCA assay

The analysis of PCA was conducted as described previously [13]. Briefly, HL-60 cells or freshly isolated by Ficoll Paque gradient peripheral blood mononuclear cells (PBMCs) were concentrated to 3×10^6 cells/ml and either untreated or treated with positive control, or test nanoparticles at 37°C in the presence of 5% CO_2. Incubation time for PBMCs and HL-60 cells were 24 and 5 h, respectively. LPS and doxorubicin (Adriamycin-TM) were used as positive controls for PBMC and HL-60, respectively. We used incubation times that have been used by others for the respective cell line and respective treatment [12,13,19,20,32]. We verified in preliminary experiments that 24 h exposure of PBMCs to LPS and 5 h exposure of HL-60 to doxorubicin results in maximum PCA response in these individual cell models in response to the positive treatment. We have also verified during preliminary experiments that shorter (5 h) incubation of PBMCs with doxorubicin or longer (24 h) incubation of HL-60 with LPS results in no significant change in the PCA induction by these agents in these cell models. For the cotreatment experiments, dendrimers and endotoxin were suspended in complete cell culture media individually and added to PBMC cultures concurrently. At the end of incubation time, cells were washed twice with Hank's Balanced Salt Solution (HBSS) containing Ca^{2+} and Mg^{2+}, and resuspended in Buffer A (20 mM HEPES, pH 7.4; 0.15 M NaCl; 6.6 mM $CaCl_2$) prewarmed to 37°C. A total of 100µl of the cell suspension was then combined with 100 µl of human plasma pooled from at least three healthy donors and anticoagulated with sodium citrate. Plasma coagulation was started by addition of post-treatment washed cells into plasma. Plasma coagulation time was measured using StarT4 coagulometer. Each sample was analyzed in two duplicates. Percentage coefficient of variation between individual replicates was within 10%, otherwise the test sample was reanalyzed.

Neoplastin™ reagent was used as an internal control to validate instrument performance. PCA of test samples was calculated as the percentage of that induced by 50 µg/ml of doxorubicin in the HL-60 model and percentage of that induced by 1 µg/ml of LPS for PBMCs.

Results

Characterization of commercial formulations of PAMAM dendrimers

Since this study attempted to map PCA induction to dendrimer physicochemical properties, it was necessary to thoroughly characterize the dendrimers before conducting the study. Understanding particle physicochemical properties and quantifying endotoxin are generally important biological studies using nanoformulations, since nanomaterial physicochemical properties may change with time and environment [33]. We measured the dendrimers' hydrodynamic sizes, zeta potentials and levels of endotoxin. The size of generation 3 (G_3) dendrimers was between 3.0 and 3.5 nm for all tested surface modifications, that of G_4 dendrimers was between 3.8 and 4.5 nm, G_5 between 5.1 and 5.9 nm, and G_6 between 6.5 and 7.6 nm (Table 1). All dendrimers with amine surface functionality had positive charge (zeta potentials between +34.6 and +46.2 mV), while all particles terminated with succinamic acid had negative charge (zeta potential between -25.7 and -43.3 mV) and G_3–G_5 dendrimers terminated with ethanolamine were neutral (zeta potential between -5.8 and +4.3 mV) (Table 1). In general, zeta potentials between -10 and +10 mV are considered neutral (Malvern Instruments, private communication). Amidoethanol terminated G_6 dendrimers were slightly cationic (zeta potential +14.7 mV) (Table 1).

The levels of endotoxin in the commercial formulations of PAMAM dendrimers used for this study were low and varied from undetectable to 0.4 endotoxin units (EU)/mg (Table 1).

Difference in PCA response to LPS, cytotoxic oncology drug & nanoparticles between normal & cancerous leukocytes

Normal leukocytes (PBMC model) responded as expected to endotoxin (LPS) treatment by exhibiting high levels of PCA (Figure 1A), while APL cells (HL-60 model) did not exhibit a detectable response to LPS (Figure 1B), even when the concentration of LPS was raised to 100 µg/ml (data not shown). By contrast, the cytotoxic oncology drug doxorubicin did not induce PCA in normal cells, but resulted in high levels of PCA induction in APL cells (Figure 1A & 1B). Cationic dendrimers behaved similar to cytotoxic oncology treatment in this *in vitro* test, in that the dendrimer-induced PCA was observed in leukemia cells but not in normal leukocytes (Figure 1A & 1B). The incubation time used in our experiments with two cell models was the one, which according to previous studies and our preliminary experiments, resulted in maximum PCA induction (details can be viewed in 'Materials and methods' section). Increase of incubation time in HL-60 model or decrease of incubation time in PBMC did not change the results shown in Figure 1.

Table 1. Summary of particle characterization.

Surface	Generation	Z-average size, DLS, nm (SD)	Zeta potential, mV (SD)	Endotoxin, EU/mg	Number of surface groups*	Molecular weight*
Amine ($-NH_2$)	G3	3.1 (0.1)	43.3 (2.0)	<0.5	32	6937
	G4	4.2 (0.1)	34.6 (0.1)	0.6	64	14,243
	G5	5.5 (0.1)	43.3 (0.7)	<0.5	128	28,854
	G6	7.5 (0.1)	46.2 (1.5)	0.06	256	58,076
Carboxy (-COOH)	G3	3.5 (0.1)	-25.7 (0.9)	0.3	32	10,137
	G4	4.5 (0.1)	-40.0 (1.4)	0.05	64	20,643
	G5	5.9 (0.1)	-33.8 (0.7)	0.4	128	41,654
	G6	7.6 (0.1)	-43.3 (0.5)	0.006	256	83,676
Hydroxy (-OH)	G3	3.0 (0.1)	-5.8(0.9)	<0.005	32	6969
	G4	3.8 (0.1)	-3.3 (1.2)	<0.005	64	14,307
	G5	5.1 (0.1)	4.3 (0.2)	<0.005	128	28,982
	G6	6.5 (0.1)	6.5 (0.5)	0.03	256	58,332

Particle hydrodynamic size was determined by DLS, shown is Z-average values. Zeta potential as property of particle surface charge was performed using zeta sizer. Endotoxin was measured using standard kinetic turbidity LAL assay.
**Theoretical data from manufacturer based on composition and synthesis.*
DLS: Dynamic light scattering; EU: Endotoxin units; LAL: Limulus amoebocyte lysate; SD: Standard deviation (n = 12).

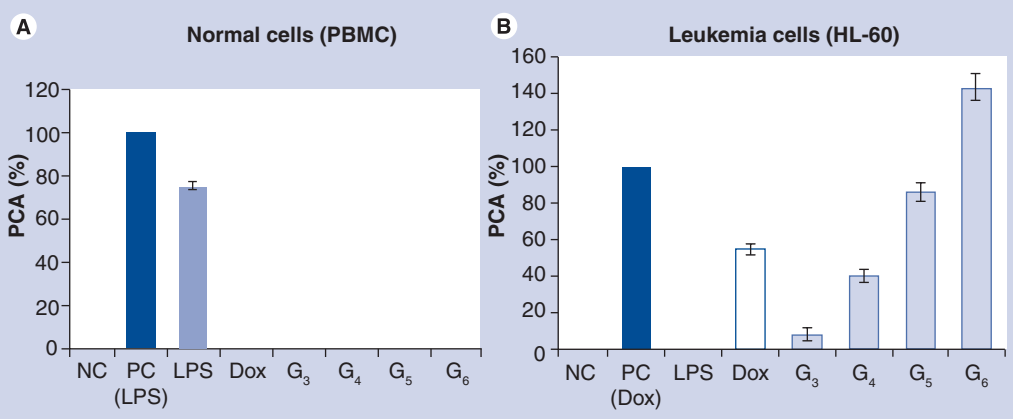

Figure 1. Difference in sensitivity to cytotoxic drugs, cationic dendrimers and endotoxin between normal and leukemia cells. PBMCs from four healthy donor volunteers **(A)** and acute promyelocytic leukemia cells (HL-60) **(B)** were treated with NC, PC, 100 ng/ml endotoxin (LPS), 25 µg/ml Dox or 50 µg/ml cationic PAMAM dendrimers (G_3, G_4, G_5 and G_6). The results shown here are the mean ± standard deviation (n = 4). PC(LPS) in PBMC was 10 µg/ml of LPS. PC(Dox) in HL-60 cells was 50 µg/ml Dox. PBS was used as negative control.
Dox: Doxorubicin; LPS: Lipopolysaccharide; NC: Negative control; PBMC: Peripheral blood mononuclear cell; PC: Positive control; PCA: Procoagulant activity.

Cationic PAMAM dendrimers enhance endotoxin induced PCA in normal leukocytes

Since cationic dendrimers did not induce PCA in normal leukocytes, we next tested whether these particles have any effect on the endotoxin-induced PCA in these cells. For this experiment, we used G_6 dendrimers because they were the most reactive in the APL cells (Figure 1B) and other blood compatibility experiments (data not shown). We treated PBMCs from individual donors with various concentrations of endotoxin, ranging from 1 pg/ml to 1 ng/ml, alone or in the presence of noncytotoxic concentrations (25 µg/ml) of G_6 amine-terminated dendrimers. Cell viability was assessed using a trypan blue exclusion test and MTT assay (data not shown).

We observed that some concentrations of endotoxin (e.g., 1.0 and 10.0 pg/ml in data shown on Figure 2) did not induce PCA in normal leukocytes when endotoxin was used alone. However, coagulation times increased significantly when these concentrations were combined with 25 µg/ml of cationic G_6 dendrimers (Figure 2). Data presented in Figure 2 were generated using cells and autologous plasma from one donor. We analyzed cells from five donors. Although we observed variability in individual donor's response to endotoxin in that some donors responded to 100 pg/ml endotoxin, while others required higher concentrations (1 ng/ml), the results of endotoxin combination with cationic dendrimers were always the same: PCA induced by endotoxin increased significantly when endotoxin treatment was combined with cationic PAMAM dendrimers (Supplementary Figure 1 see online www.futuremedicine.com/doi/suppl/10.2217/nnm.11.105). Enhancement of endotoxin-induced PCA was observed for the entire range of tested endotoxin concentrations (Figure 2 & Supplementary Figure 1).

Cationic, but not anionic & neutral PAMAM dendrimers, induce PCA in leukemia cells in a size- & surface charge-dependent manner

We next conducted a similar evaluation using promyelocytic leukemia cells. We treated these cells with amine-terminated (cationic), succinamic acid-terminated (anionic) and amidoethanol-terminated (neutral) dendrimers of four sizes – G_3, G_4, G_5 and G_6 at final concentrations of 50 µg/ml. No dendrimers were appreciably cytotoxic at this concentration according to a trypan blue exclusion test and MTT assay (data not shown). Neither the anionic or neutral dendrimers induced leukocyte PCA in leukemia cells (Table 2), but cationic dendrimers did result in the induction of the leukocyte PCA. Moreover, this induction appeared to depend on the generation of the dendrimers, with higher generations inducing greater levels of PCA (Table 2).

Since the degree of cationic dendrimer-induced PCA induction correlated with dendrimer generation, and increasing dendrimer generation is known to have increasing size and surface charge (expressed in terms of number of charged surface groups per dendrimer), we next explored if the PCA induction depended more heavily on size or charge. We tested each cationic

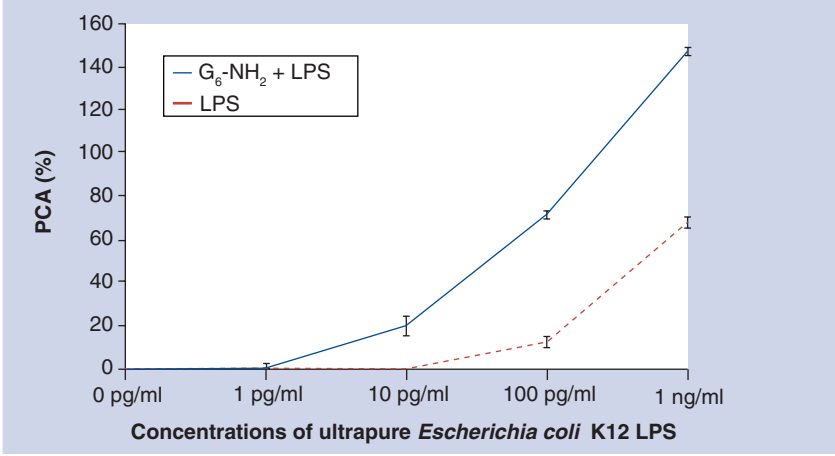

Figure 2. Generation 6 cationic PAMAM dendrimers enhance endotoxin-induced procoagulant activity in normal leukocytes. Peripheral blood mononuclear cells from five healthy donor volunteers were either left untreated or treated with endotoxin alone (red dashed line, LPS) or endotoxin in combination with 25 µg/ml of G_6 cationic PAMAM dendrimers (blue line, G_6-NH_2+LPS). The results shown here are the mean ± standard deviations (n = 2), studied using cells and autologous plasma from one donor. Similar results were obtained with cell-plasma sets from other donors (Supplementary Figure 1).
LPS: Lipopolysaccharide; PAMAM: Polyamidoamine; PCA: Procoagulant activity.

dendrimer formulation at a range of concentrations varying from 1.5625 to 100.0 µg/ml (Supplementary Figure 2), and compared particles of different generations at equivalent mass (mg/ml) concentrations (equimass) and at equivalent molar concentrations (equimolar) against the percent PCA. When equimass concentrations of G_3, G_4, G_5 and G_6 dendrimers are compared, the samples have the same total number of amines in solution (Figure 3A) [34] , whereas when equimolar concentrations are compared, the total number of amines in solution doubles with each increasing generation (i.e., G_4 as two-times as many total amines in solution as G_3, and similarly for G_5 to G_4, G_6 to G_5) (Figure 3C) [34]. Therefore, if there is a strong dependence of PCA induction on total charge, there should be a significant difference between the two comparisons. The ideal way of estimating the role of surface charge as it relates to the number and density of surface amines is the use of the dendrimers of the same generation in which a certain number of surface amines are neutralized. But since such dendrimer samples are not available commercially, we have used the indirect approach of comparing particles of various generations at equimolar concentration.

When the percentage of PCA induction was plotted versus equimass (50.0 µg/ml) concentrations of G_3, G_4, G_5 and G_6 dendrimers, the percentage of PCA induced in leukemia cells by the cationic dendrimers increased for each generation (Figure 3B) and it also increased similarly when plotted versus equimolar (0.9 µM) concentrations (Figure 3D).

Table 2. *In vitro* procoagulant activity of polyamidoamine dendrimers.

Generation	Terminal functional group		
	-NH_2	*-COOH*	*-OH*
G_3	10.7 ± 4.6	0	0
G_4	27.2 ± 12.8	0	0
G_5	44.2 ± 21.9	0	0
G_6	82.8 ± 15.5	0	0

Acute promyelocytic leukemia cells, HL-60, were either left untreated or treated with various generations of polyamidoamine dendrimers at concentrations of 50 µg/ml. The values shown here are the mean ± standard deviations (n = 4). Procoagulant activity is expressed as percentage of that induced by 50 µg/ml of doxorubicin, which was used as a positive control.

Cationic dendrimer-induced PCA requires *de novo* RNA & protein synthesis, & is dependent on both phospholipid & tissue factor

Since previous studies demonstrated that doxorubicin-induced PCA in leukemia requires *de novo* RNA and protein synthesis, and is dependent on both lipid and protein (tissue factor) components [13], we next tested whether the same applies to cationic dendrimer-induced PCA. The purpose of this experiment was to see whether or not cationic dendrimers share the mechanism of PCA induction with cytotoxic oncology drugs or if dendrimer-induced PCA induction occurred via a separate, 'nano-specific' mechanism. To gain insight into the mechanism of the dendrimer-mediated PCA induction in leukemia cells, we used a series of several different inhibitors. We treated APL cells (HL-60 cells) with doxorubicin, and cationic G_3–G_6 dendrimers, alone or in combination with actinomycin D (an RNA synthesis inhibitor), cycloheximide (a protein synthesis inhibitor), phospholipase C (surface phospolipid expression inhibitor) and a neutralizing antibody against tissue factor. The named inhibitors were used because their utility in studying PCA expression by leukocytes has been established before [12,13,19,35]. The preference to using TF neutralizing antibody over traditional RT-PCR was given due to complex postransriptional regulation of this protein, including the encryption/de-encryption process [36]. Doxorubicin-induced PCA was inhibited by all these inhibitors (Figure 4). Similarly to doxorubicin, cationic dendrimer-induced PCA was dependent on RNA and protein synthesis (Figure 4A & 4B, respectively). It was also inhibited by phospholipase C and antibody neutralizing tissue factor (Figure 4C & 4D, respectively).

Discussion

Nanoparticle size and surface charge are currently recognized as important physicochemical properties determining nanoparticle biocompatibility [37]. These two properties were named critical nanoscale design parameters, which may constitute predictive 'nano-periodic property patterns', and therefore influence toxicity of dendrimers [38]. To understand the relationship between particle physicochemical properties, such as size and surface charge, and their ability to induce leukocyte PCA, we studied 12 formulations of PAMAM dendrimers. These dendrimers were selected to allow comparison of nanoparticles with similar sizes but different surface charges, and similar charges but different sizes. The study included four generations (G_3, G_4, G_5 and G_6), and three surface functionalities (succinamic acid, amine and amidoethanol). It has been reported earlier that neutral, cationic and anionic dendrimers can intercalate into a lipid bilayer, but only cationic dendrimers can induce formation of holes in the cell membrane [39–42]. This mechanism was suggested to explain cytotoxicity of cationic dendrimers [39,40]. To avoid observing differences between the formulations, which are due to dendrimer cytotoxicity, we have tested all formulations at noncytotoxic concentrations.

Since this study sought to map PCA induction to dendrimer physicochemical properties (e.g., size and charge), it was necessary to thoroughly characterize the dendrimers' physicochemical properties before conducting the study. Dynamic light scattering is a useful way to obtain information about the hydrodynamic size (diameter) of a nanoparticle in solution [33]. The zeta potential is the measure of net charge on the particle. Since nanoparticle zeta potential is related to its surface charge, it has been shown to play a role in nanoparticle uptake and plasma–protein binding, and may be important for endotoxin adherence [33,43,44]. Particle size and zeta potential were measured in physiologically relevant buffers (PBS and 10 mM NaCl solution, respectively); analysis of these properties in the presence of serum proteins was not possible due to technical limitations of dynamic light scattering and zeta sizer, which resulted from inability of the instruments to distinguish between proteins and protein-like, water-soluble polymeric particles [45]. Both the size and zeta potentials of the dendrimers were within what is expected theoretically and reported by the supplier for all formulations. Zeta potential is a good technique for distinguishing between cationic, anionic and neutral particles. However, it does not provide a quantitative measurement of surface charge within each particle category in that it cannot be used to distinguish more cationic particles from less cationic [46,47]. This is why we relied on zeta potential when we compared the anionic,

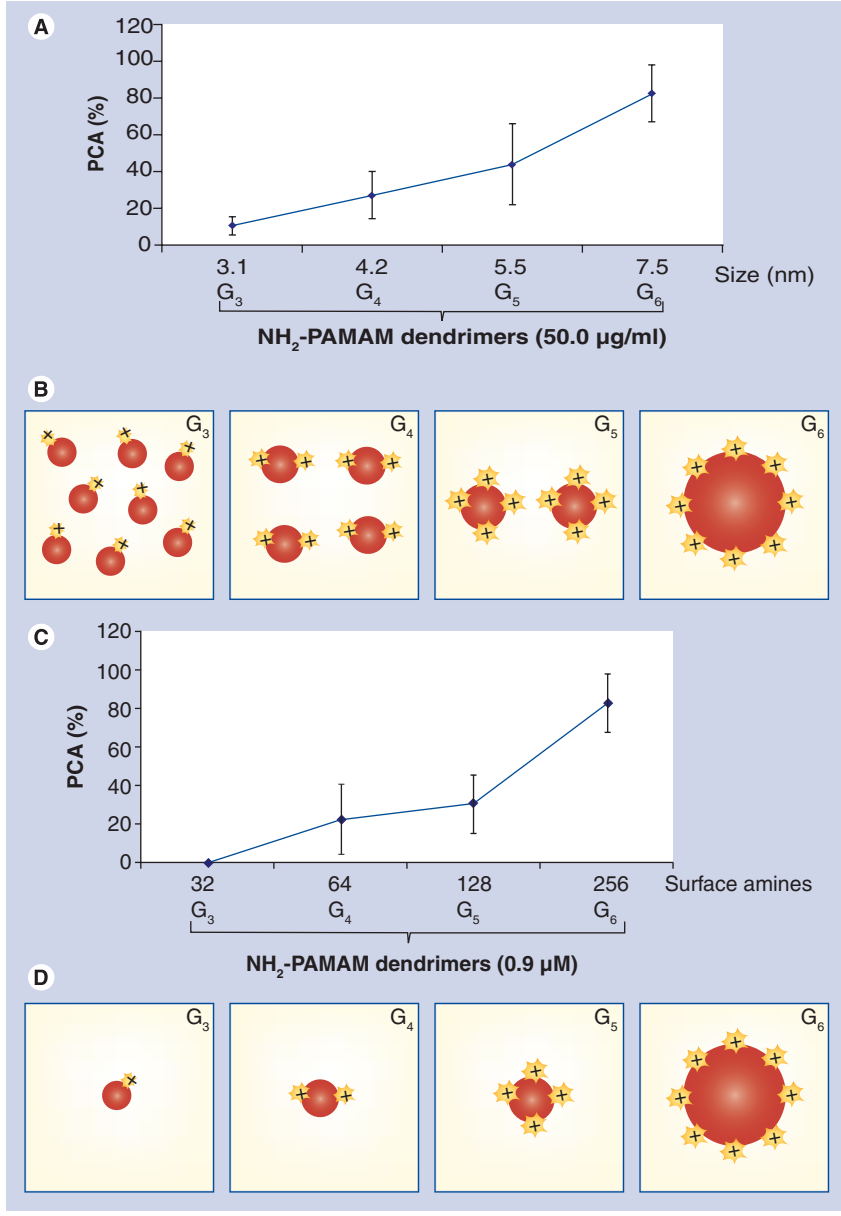

Figure 3. Cationic polyamidoamine dendrimer-induce leukocyte procoagulant activity in leukemia cells in a size- and surface charge-dependent manner. HL-60 cells were either left untreated or treated with cationic G_3, G_4, G_5 or G_6 PAMAM dendrimers at a concentration range from 100.0 to 1.5625 µg/ml. Each result shown here is the mean ± standard deviation (n = 6). Doxorubicin (50 µg/ml) was used as positive control. Percentage PCA was calculated for each concentration as percent of that induced by the positive control. **(A & B)** Effects of size: each generation (G_3, G_4, G_5 and G_6) was compared at equivalent mass concentration (50.0 µg/ml). **(C & D)** Effects of surface charge as related to number of surface amines: each generation (G_3, G_4, G_5 and G_6) was compared at equivalent molar (0.9 µM/ml) concentration. **(B & D)** Star with '+' symbolizes surface amines.
LPS: Lipopolysaccharide; PAMAM: Polyamidoamine; PCA: Procoagulant activity.

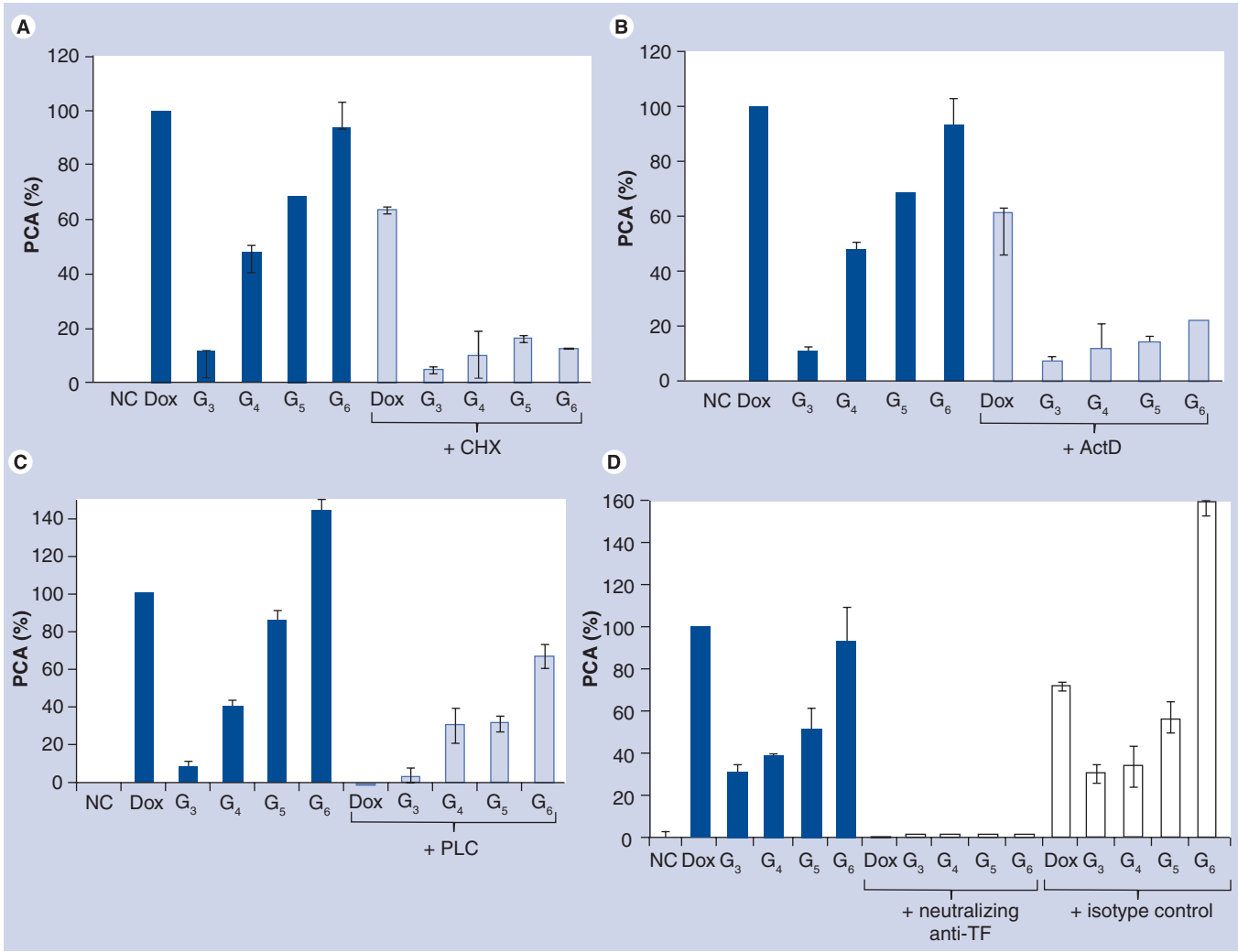

Figure 4. Insight into mechanisms of the dendrimer-induced procoagulant activity. APL cells (HL-60 cells) were either left untreated or treated with Dox, or cationic PAMAM dendrimers (G_3, G_4, G_5 or G_6) at a final concentration of 50.0 µg/ml alone or in combination with **(A)** 5 µg/ml of CHX; **(B)** 3 µg/ml of ActD; **(C)** 10 µg/ml of PLC; and **(D)** 10 µg/ml of neutralizing TF antibody. The results shown here are the mean ± standard deviation (n = 4). Dox (50 µg/ml) was used as positive control. Percentage PCA was calculated for each concentration as percent of that induced by the positive control.
ActD: Actinomycin D; CHX: Cycloheximide; Dox: Doxorubicin; NC: Negative control; PBMC: Peripheral blood mononuclear cell; PCA: Procoagulant activity; PLC: Phospholipase C.

neutral and cationic categories, and we relied on the number of surface groups rather than zeta potential when we compared the effects of the surface charge within individual categories.

Since endotoxin contamination of nanomaterials has been linked to erroneous conclusions in immunological tests [48,49], we have also determined the amounts of endotoxin in the studied formulations. The LAL test detected negligible levels of endotoxin in all 12 formulations. For example, the highest endotoxin level in this study was detected in the G_4 amine-terminated dendrimers (0.6 EU/mg). At the highest dendrimer concentration used in our experiments, this would still correspond to below 0.03 EU/ml (or 3.0 pg/ml) in cell culture media. Our data in both endotoxin responsive cells (PBMCs), and leukemia cells known to be insensitive to endotoxin, suggest that endotoxin levels below 10 pg/ml do not produce false-positive results. For the purpose of this study endotoxin 'sensitivity' refers to the cell's ability to respond to endotoxin by expressing leukocyte PCA.

We next performed qualification of the *in vitro* test for leukocyte PCA using PBMCs and leukemia cells (APL cells, HL-60 cells) to make sure our results testing doxorubicin and endotoxin in these cells agreed with previously published data [13,22,32]. Our results confirmed that endotoxin induces PCA induction in normal leukocytes (PBMCs) and doxorubicin does so in the leukemia cells (APL cells, HL-60 cells), in agreement with the previously published literature [13,22,32,50].

Interestingly, cationic dendrimers performed similarly to cytotoxic oncology drugs (e.g., doxorubicin) in this *in vitro* assay, in that they did not induce PCA in normal cells but did induce PCA in leukemia cells (Figure 1). The tests with PBMCs also revealed an interesting property of the dendrimers: although dendrimers alone did not induce PCA in normal cells, they enhanced endotoxin-induced PCA induction (Figure 2). The mechanism(s) of this enhancement has not been established. Since in our experiments the dendrimers were added to cell cultures concurrently with endotoxin, one such mechanism could be electrostatic interaction between cationic dendrimer and anionic endotoxin resulting in local increase in endotoxin concentration available for interaction with endotoxin receptor. More studies have to be completed to verify this hypothesis. There is an increasing number of studies reporting nanoparticles' ability to enhance inflammatory properties of endotoxin [51,52] and endotoxin's ability to promote nanoparticle inflammatory properties [53]. Therefore, nanoparticle interactions with endotoxin become an increasingly important issue. The results of our study point to a potential issue that is important for nanomedicine developers to consider; since amines have greater chemical reactivity than hydroxyl or carboxy groups, cationic dendrimers are usually a more attractive candidate for attachment of drugs and targeting moieties. For example, nucleic acid delivery therapies generally require a cationic carrier. Our data suggest that in order to avoid coagulopathy complications with cationic dendrimers, one should consider designing the prodrug systems such that the carrier (dendrimer in this case) is neutral after the drug is released and the formulation is endotoxin free. That is, free amines should be passivated, since any free amines can enhance response to the endotoxin. Since other cell types (e.g., endothelial cells) are also known to express PCA [17,18], it would be interesting to understand whether the findings described in our study for leukocytes are applicable to other types of cells also involved in maintaining hemostasis.

We next focused on the leukemia cell model (HL-60 cells) to study the relationship between dendrimer structure and their ability to induce leukocyte PCA. We also used this model to look at mechanisms of dendrimer-induced PCA induction. We showed that neutral and anionic dendrimers do not induce PCA, while cationic dendrimers are reactive in this test (Table 2). The PCA induction increased with increasing generation (G_3<G_4<G_5 <G_6). Since higher dendrimer generations have both larger sizes, greater molecular weight and surface charges, this suggested that either particle size or particle surface charge (or both) are important modulators of this effect. To further investigate these parameters we tested cationic dendrimers at a range of concentrations, and analyzed the data in two ways: comparing PCA response against equivalent particle mass concentration (equimass concentrations), and comparing PCA response against equivalent particle molar concentrations (equimolar concentrations). Interestingly, for the PAMAM dendrimers, the molecular weight and the number of terminal groups double with each increasing generation resulting in an equal number of total terminal groups when equal mass amounts of each generation dendrimers are tested. At equimass concentrations, we observed an approximately twofold difference in percentage of PCA between the generations (Figure 3A), and at equimolar concentrations, we also saw an increase in percentage of PCA (Figure 3B).The similarity of these results indicates that there is no strong dependence of PCA on total charge (total number of surface amines) in solution.

Since cationic dendrimers are known to be cytotoxic [54] and have been reported as DNA-binding agents [55], we hypothesized that they might induce PCA via the same mechanism as cytotoxic, DNA-binding drugs, such as doxorubicin. Indeed, we observed similar results with the dendrimers as with doxorubicin, in that both doxorubicin-induced PCA and dendrimer-induced PCA in leukemia cells were inhibited by RNA synthesis and protein synthesis inhibitors, as well as by phospholipase C and by a neutralizing antibody of tissue factor (Figure 4).

Conclusion

Our study demonstrated that PCA induction depended on particle size and surface charge, in that not anionic or neutral, but large cationic dendrimers and those with greater number of surface amines induced greater PCA. The density of these amines on the particle surface may contribute towards the PCA induction. We showed that the mechanism of PCA induction by cationic dendrimers was similar to that of doxorubicin. A correlation between *in vitro* PCA of cytotoxic oncology drugs, including doxorubicin, has previously been shown to correlate with thrombogenic complications, such as DIC *in vivo* [3]. Since cationic PAMAM dendrimers appear to share an *in vitro* PCA induction mechanism with cytotoxic oncology drugs, one can

expect that cationic dendrimers may cause a similar toxicity (e.g., DIC) *in vivo* as those described for cytotoxic oncology drugs.

We also demonstrated that cationic PAMAM dendrimers do not induce leukocyte PCA in normal leukocytes, but do enhance PCA induced by endotoxin. Since endotoxin is known to induce DIC complications in patients, we speculate that endotoxin contamination of some nanomaterials (such as cationic PAMAM dendrimers) may exaggerate this known toxicity of endotoxin.

In summary, since dendrimers are being considered as drug-delivery platforms, our data caution that surface amines have to be covered or 'masked' from immune cell recognition after the drug release, and the formulations must be endotoxin free in order to avoid thrombogenic complications associated with enhanced leukocyte PCA.

Future perspective

While our study confirms the role of tissue factor in dendrimer-induced PCA, the exact nature of PCA induction by the dendrimers has yet to be determined. For example, TF expression is a very complex process. The protein is shown to undergo post-translational regulation described in the current literature as 'encryption' process [36]. Our data point to the necessity of the *de novo* RNA and protein synthesis, and highlight the function of the TF protein; however, it is not clear at this point whether dendrimers induce TF expression directly, or they induce other secondary messengers responsible for the TF gene induction. It is also unknown whether and how dendrimers contribute to the de-encryption of TF protein to make it a functional component of the PCA on the cell surface. More detailed mechanistic studies are necessary to answer these questions.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Cationic, but not anionic or neutral, polyamidoamine (PAMAM) dendrimers stimulate leukocyte procoagulant activity (PCA) of leukemia cells *in vitro*. This property is dependent on particle size and charge, in that the percentage of PCA increases with dendrimer generation.
- The mechanism of leukocyte PCA induction by cationic PAMAM dendrimers in leukemia cells appears to share a mechanism with that described for cytotoxic, DNA-intercalating oncology drugs, in that it requires *de novo* RNA and protein synthesis, and is dependent on lipid and protein (tissue factor) components.
- Cationic PAMAM dendrimers do not induce procoagulant activity of normal leukocytes; however, they enhance PCA induced by bacterial endotoxin in these cells.
- The data obtained in this study suggest that when cationic dendrimers are used as drug-delivery platforms, they may require careful design to cover cationic moieties after drug release. Additionally, special care should be taken to ensure the formulations are endotoxin-free in order to avoid thrombogenic complications caused by cationic dendrimer-mediated enhancement of endotoxin-induced PCA.

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