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Mechanism of cell death induced by cationic dendrimers in RAW 264.7 murine macrophage-like cells

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Abstract

Cationic dendrimers possess attractive nano-sized architectures that make them suitable as targeted drug/gene delivery systems. However, very little is known about their mechanisms of cell death in cellular systems. In the current study, the apoptotic and necrotic effects of starburst polyamidoamine (PAMAM) and polypropylenimine (DAB) dendrimers in cultured RAW 264.7 murine macrophage-like cells were investigated. Cationic dendrimer treatment produced a typically dose-dependent cytotoxic effect on macrophage cells. RAW 264.7 cells exposed to cationic dendrimers exhibited morphological features of apoptosis. Apoptotic ladders were observed in DNA extracted from RAW 264.7 cells treated with cationic dendrimers. Analysis from flow cytometry demonstrated an increase in hypodiploid DNA population (sub-G1) and a simultaneous decrease in diploid DNA content, indicating that DNA cleavage occurred after exposure of the cells to cationic dendrimers. Also, cells treated with DAB dendrimer induced a higher percentage of sub-G1 population than those treated with PAMAM dendrimer at the same dose. In addition, it was shown that pre-treatment of RAW 264.7 cells with the general caspase inhibitor zVAD-fmk prevented some degree of apoptosis induced by cationic dendrimers, suggesting that apoptosis in macrophage cells involves a caspase-dependent pathway. Macrophage cells were also found to be sensitive to induction of apoptosis by dendrimers, whereas NIH/3T3 cells (mouse fibroblast) and BNL CL.2 (mouse liver) cells did not undergo apoptosis. These results could be helpful for optimizing the biocompatibility of dendrimers used for targeted drug/gene delivery.

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Introduction

Cationic dendrimers are a family of highly branched polymers characterized by their unique structure and properties (Tomalia 1995; Florence & Hussain 2001; Boas & Heegaard 2004). The attractive nano-sized architectures of cationic dendrimers make them suitable for many pharmaceutical applications such as delivery of DNA and oligonucleotides into eukaryotic cells, solubilization of sparingly soluble drugs, nanocarriers for transepithelial transport, and platforms for cancer therapeutics (Haensler & Szoka 1993; Pistolis et al 1999; Esfand & Tomalia 2001; Cloninger 2002; Dennig & Duncan 2002; Zinselmeyer et al 2002). A variety of cationic dendrimers, such as starburst polyamidoamine (PAMAM), polypropylenimine and phosphorus-containing dendrimers, have been studied as potent gene delivery systems (Haensler & Szoka 1993; Zinselmeyer et al 2002; Maksimenko et al 2003). Previous studies have demonstrated that cationic PAMAM can permeate across Caco-2 cell monolayers, suggesting their potential as oral delivery systems (El-Sayed et al 2002; Jevprasesphant et al 2004). A key consideration in the development of these delivery systems will be the toxicity of cationic dendrimers used. Since cationic dendrimers are positively charged, the toxicity of such polycations might increase from a direct interaction with biological membranes (Morgan et al 1989; Choksakulnimitr et al 1995; Fischer et al 2003). It has been shown that cationic dendrimers can induce cell death in-vitro and in-vivo (Brazeau et al 1998; Malik et al 2000). In another report, PAMAM dendrimers (generations 3 and 5) did not exhibit in-vivo cytotoxicity in mice (Roberts et al 1996).

There are two major mechanisms by which cells die in biological systems: necrosis and apoptosis (Majno & Joris 1995). Necrosis is related to inflammatory and degenerative

processes. Cells undergoing necrosis characteristically demonstrate mitochondrial swelling, loss of membrane integrity, metabolism shut down, and release of cytoplasmic components, stimulating an inflammatory response (Bianchi & Manfredi 2004). In contrast to necrosis, apoptosis, or shrinkage necrosis, is a form of programmed cell death that is characterized by cytoplasmic blebbing, condensation of the nuclear chromatin, cell shrinkage, DNA fragmentation, exposure of phosphatidylserine residues on the outer leaflet, and cellular fragmentation into membrane apoptotic bodies (Kerr et al 1972; Cummings et al 1997). To date, only one research group has shown that the cytotoxicity induced by PAMAM dendrimers in hepatocytes was characterized by apoptosis (Kawase et al 2001; Higashiyama et al 2003). Therefore, a better understanding of the mechanisms of cell death induced by cationic dendrimers is necessary for the further development of appropriate delivery systems.

Dendrimers are primarily captured by the mononuclear phagocyte system following intravenous injection (Kawakami et al 2000). Therefore, this characteristic is beneficial for efficient delivery of antigens or immunomodulating agents to macrophages and triggering specific immune response (Foged et al 2004). Also, a recent study has shown that induction of apoptosis and necrosis by vaccine adjuvants in mouse thymoma cells is critical for antigen processing and presentation (Wu & Yang 2004).

In the present study, the murine macrophage-like cell line RAW 264.7 was selected as a model cell line to represent the physiological scavengers of foreign nanoparticles in the body. The aim of the study was to provide a basic understanding of the changes that occur during different mechanisms of cell death induced by dendrimers. These insights into the mechanism of cell death could be helpful for optimizing the biocompatibility of dendrimers used for targeted drug/gene delivery.

Materials and Methods

Materials

Starburst PAMAM (generation 5) and polypropylenimine (DAB 5.0) dendrimers were purchased from Aldrich Chemicals (St Louis, MO, USA). Starburst PAMAM was supplied in methanol, which was removed by vacuum evaporation, and stored with phosphate-buffered saline (PBS) at 4°C. The general caspase inhibitor zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was obtained from Promega Corporation (Madison, WI, USA). Propidium iodide (PI) was from Sigma (St Louis, MO, USA).

Cells

The murine macrophage-like cell line RAW 264.7 was maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 U mL^{-1} penicillin/100 μg mL^{-1} streptomycin (Sigma).

Cytotoxicity assays

RAW 264.7 cells were seeded in 96-well plates at 20 000 cells/100 μL/well and incubated for 1 day at 37°C and 5% CO_2. Positive control cells were grown without adding cationic dendrimers. Then, 10 μL of various concentrations of cationic dendrimers was added to the culture medium and incubated for 24 h. To measure the cell viability, 10 μL of the Cell Counting Kit-8 solution (a tetrazolium salt that produces a highly water-soluble formazan dye upon biochemical reduction in the presence of an electron carrier (1-methoxy PMS; Dojindo Laboratories, Tokyo, Japan) was added to each well and incubated for 1–4 h. The amount of the yellow coloured formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells in a culture medium. The absorbance at 450 nm was obtained using an ELISA reader with a reference wavelength at 595 nm. Results are reported as % cell viability (average optical density/average positive control optical density) ± s.d.

Lactate dehydrogenase (LDH) release assays

LDH activity was assessed using the LDH cytotoxicity detection kit from Takara Bio Inc. (Otsu, Shiga, Japan). In brief, 5000 RAW 264.7 cells/200 μL/well were added with various concentrations of dendrimers and then incubated for 1 day at 37°C and 5% CO_2. After incubation, 100 μL/well supernatant was carefully removed and transferred into an optically clear 96-well plate. Then, 100 μL of reaction solution provided by the manufacturer was added to each well and incubated for 30 min in the dark. The enzyme reaction was stopped by the addition of 1 M HCl at 50 μL/well. The absorbance at 490 nm was measured using an ELISA reader with reference wavelength at 620 nm. The relative LDH release was calculated by the ratio of LDH release over control samples. Controls were treated with 1% Triton X-100 and set as 100% LDH release and 0% for cells with culture medium only.

PI nuclear staining

Untreated and dendrimer-treated RAW 264.7 cells (2×10^5) were incubated for 24 h and harvested. The cells were fixed with 1% paraformaldehyde for 60 min at room temperature and washed three times with 0.1% Tween 20 in PBS. After washing, the cells were incubated with PI staining solution (40 μg mL^{-1} PI, 100 μg mL^{-1} RNase A) for 30 min in the dark. The cells were washed five times with PBS, and then viewed under a fluorescent microscope (Axiovert S100; Zeiss, Gottingen, Germany).

DNA fragmentation assays

RAW 264.7 cells (1×10^6) treated with dendrimers for 24 h were washed twice with PBS, suspended in 500 μL of lysis buffer (20 mM Tris, 10 mM EDTA, 0.2% TritonX-100, pH 8.0), and incubated on ice for 10 min. After centrifugation at 437 g for 10 min, the cell lysate was incubated with proteinase K (Sigma; final concentration 200 μg mL^{-1}) to digest protein at 50°C for 8 h and then further incubated

with RNase A (Sigma; final concentration 100 μg mL^{-1}) to digest RNA at 37°C for 6 h. DNA was extracted twice using saturated phenol solution and followed by chloroform/isoamylalcohol extraction. After centrifugation at 4370 g for 10 min, glycogen (Sigma; final concentration 20 μg mL^{-1}) and an equal volume of isopropanol were added to the upper aqueous layer. After storage at −20°C for 24 h, the extracted DNA was then dissolved in TE buffer solution (10 mM Tris, 1 mM EDTA, pH 8.0) and subjected to 2% agarose gel electrophoresis at 100 V for 30 min. The experiments were repeated more than three times to ensure the reproducibility of the assays.

DNA content

Untreated and dendrimer-treated cells (1×10^6) were fixed with fixation solution (70% ethanol, 30% PBS) at 4°C for at least 24 h. The cells were then centrifuged at 437 g for 10 min to remove fixation solution. The cell pellets were incubated with DNA staining solution (40 μg mL^{-1} PI, 100 μg mL^{-1} RNase A) for 30 min in the dark. The cells were analysed by flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA, USA). A total of 10 000 cells per sample were analysed and each experiment comprised six replicates.

zVAD-fmk inhibition

Cells (1×10^6) were either pre-treated or untreated with 50 μM zVAD-fmk at 37°C for 6 h, followed by treatment with cationic dendrimers at 37°C for 24 h. Cells were then stained with PI fixation solution as described above and analysed by flow cytometry.

Statistical analysis

Results are given as means ± s.d. of six replicates. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's test. Data were considered to differ significantly when $P < 0.05$.

Results

We investigated the interaction of cationic dendrimers, starburst PAMAM and DAB dendrimers, in macrophage cellular systems. The effects of cationic dendrimers on cytotoxicity, cell membrane integrity, cell morphology, DNA fragmentation, DNA content and zVAD-fmk inhibition were evaluated. We found that cationic dendrimers induced apoptosis in the murine macrophage-like cell line RAW 264.7.

Cell viability assays

Figure 1A shows that cell viability was dramatically reduced with increasing concentrations of PAMAM and DAB dendrimers. Also, cationic dendrimers have a very low inhibitory concentration 50% (< 10 μg mL^{-1}; Figure 1A), indicating that macrophages are very sensitive to the toxic effects of cationic dendrimers. This is consistent with the findings of a previous study in which macrophages demonstrated greater sensitivity than other tested cultured cells (Choksakulnimitr et al 1995). For the LDH release assays (Figure 1B), similar results were observed in dendrimer-treated cells, which showed a concentration-dependent decrease of membrane integrity after the addition of cationic dendrimers, consistent with previous reports (El-Sayed et al 2002; Jevprasesphant et al 2003a, b).

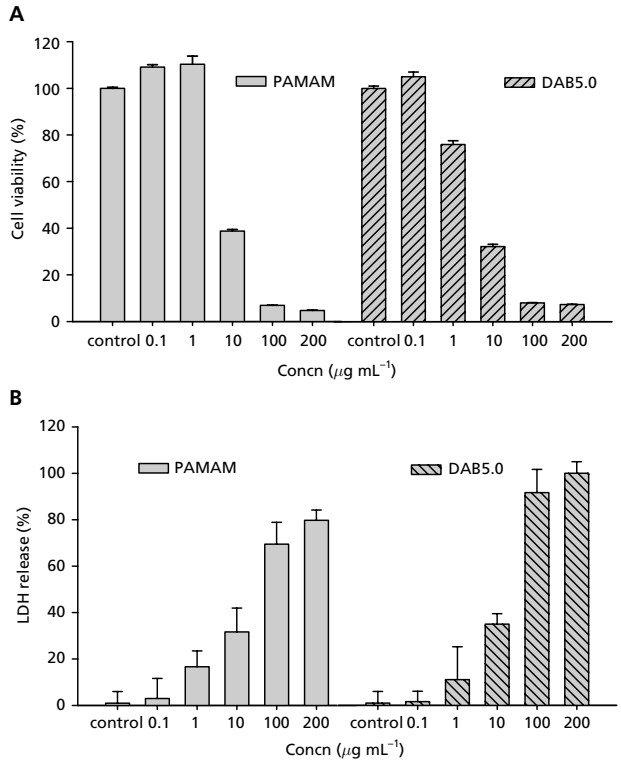

Figure 1 A. Cytotoxicity assays measuring generated dehydrogenases. Positive control cells were grown without adding cationic dendrimers. Results are reported as % cell viability (average optical density/average positive control optical density) ± s.d., n = 6. B. Lactate dehydrogenase (LDH) release. The relative LDH release was calculated by the ratio of LDH release over control samples. Controls were treated with 1% Triton X-100 and set as 100% LDH release and 0% for cells with culture medium only. Results were given as means ± s.d., n = 6.

Morphology changes of RAW 264.7 cells induced by dendrimer treatment

The hallmarks of apoptosis such as chromatin condensation and cell shrinkage developed after the addition of dendrimers, whereas the nuclei of untreated cells were more homogenously stained and less intense than those of dendrimer-treated cells (Figure 2).

DNA fragmentation assays

In cells undergoing apoptosis, a typical ladder pattern occurs due to the activation of endogenous endonuclease and produces DNA fragments in multiples of about

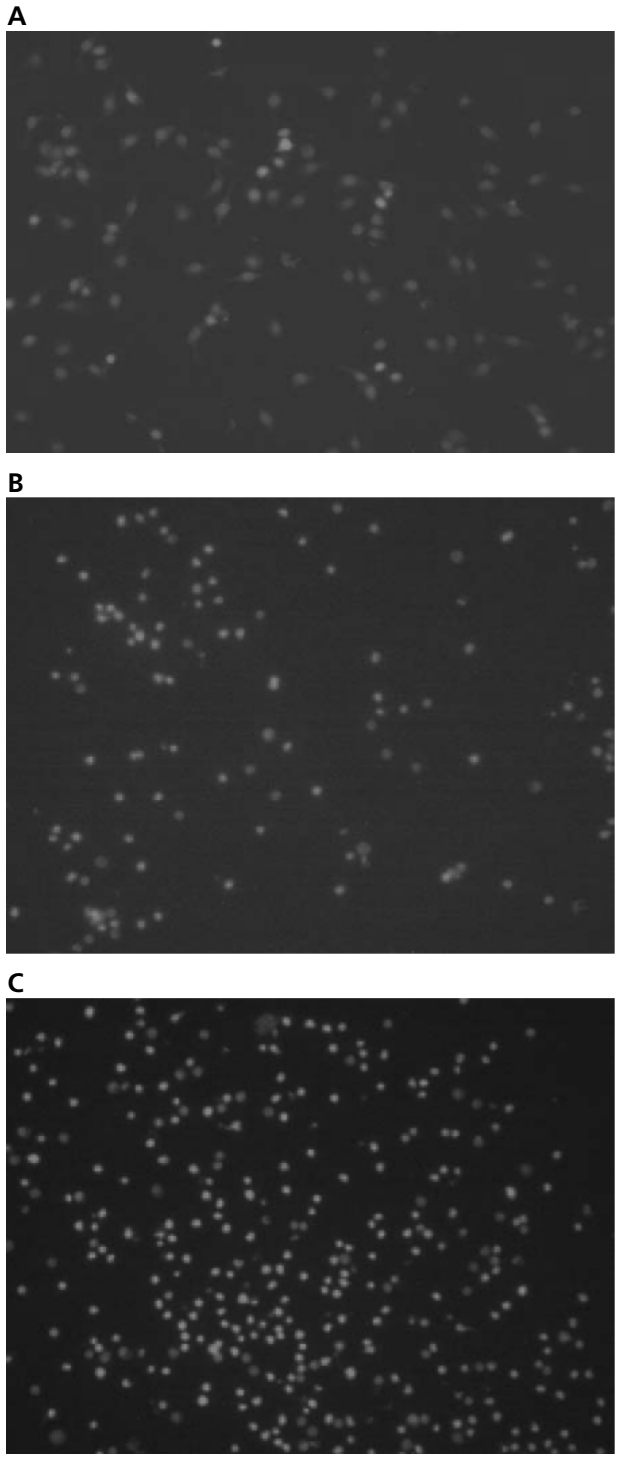

Figure 2 Morphology changes of RAW 264.7 cells induced by cationic dendrimer treatment for 24 h. Untreated cells (A) were compared with cells treated with 10 μg mL^{-1} PAMAM dendrimer (B) and 10 μg mL^{-1} DAB dendrimer (C) (200×).

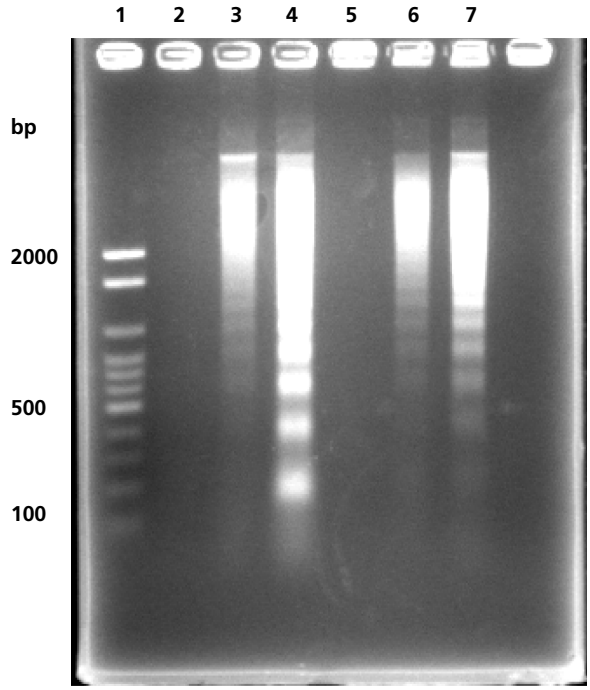

Figure 3 Agarose gel electrophoresis of DNA extracted from RAW 264.7 cells treated with cationic dendrimers for 24 h. Lane 1: DNA marker; lane 2: control (untreated cells); lane 3: 10 μg mL^{-1} PAMAM dendrimer treatment; lane 4: 15 μg mL^{-1} PAMAM dendrimer treatment; lane 5: control (untreated cells); lane 6: 10 μg mL^{-1} DAB dendrimer treatment; lane 7: 15 μg mL^{-1} DAB dendrimer treatment.

180–200 base pair units (Majno & Joris 1995). As shown in Figure 3, the typical DNA ladders were clearly visible in PAMAM and DAB dendrimers-treated cells. As the concentration of PAMAM and DAB dendrimers increased from 10 to 15 μg mL^{-1}, the relative brightness of DNA ladders also increased. From these observations, PAMAM and DAB dendrimers induced apoptosis in RAW 264.7 cells.

DNA content

A cell varies between hypodiploid and diploid DNA during the cell cycle and flow cytometry can be used to determine its position in the cell cycle based on its DNA content. In the present study, a single laser (linear PI fluorescence, 488 nm) flow cytometer was used for determining DNA strand breaks in the dendrimer-treated cells. PI-stained cells after treatment with PAMAM and DAB dendrimers showed an increase in hypodiploid DNA and a simultaneous decrease in diploid DNA content, indicating that DNA cleavage occurred after exposure of the cells to cationic dendrimers (Table 1). Also, cells treated with DAB dendrimer induced a greater percentage of sub-G1 population than those treated with PAMAM dendrimer at the same dose. This indicated that the DAB dendrimer induced more apoptotic cells than the PAMAM dendrimer.

zVAD-fmk inhibition

As shown by flow cytometry, PI-stained cells pre-treated with zVAD-fmk followed by the addition of PAMAM

Table 1 Analysis of DNA content of RAW 264.7 cells after treatment with cationic dendrimers, PAMAM and DAB, for 24 h

	[sub-G1/M1] (%)
Untreated	4.23 ± 2.01
10 $\mu g\,mL^{-1}$ PAMAM	24.72 ± 2.35
15 $\mu g\,mL^{-1}$ PAMAM	26.43 ± 1.82
10 $\mu g\,mL^{-1}$ DAB	46.38 ± 2.52
15 $\mu g\,mL^{-1}$ DAB	56.03 ± 2.15
15 $\mu g\,mL^{-1}$ PAMAM + pre-incubation with 50 μM zVAD-fmk	12.83 ± 2.14
15 $\mu g\,mL^{-1}$ DAB + pre-incubation with 50 μM zVAD-fmk	20.35 ± 1.63

Results are given as mean ± s.d., n = 6. M1 = sub-G1 + G0/G1 + S + G2/M.

and DAB dendrimers demonstrated an increase in diploid DNA content and a decrease in sub-G1 peak (Table 1). This indicates that apoptosis induced by cationic dendrimers can be inhibited to some degree by pre-treatment with zVAD-fmk, suggesting that a caspase-dependent pathway may be involved. When the concentration of zVAD-fmk was increased to 100 μM, no further inhibitory effect was observed for dendrimer-treated cells (data was not shown). Also, the inhibitory effect of zVAD-fmk was more profound in cells treated with DAB dendrimer.

Discussion

Cationic dendrimers may be potential candidates for use as vaccine adjuvants to stimulate subsequent humoral and cellular immunity (Gregoriadis et al 1997; Singh et al 2000; Regnstrom et al 2003). However, the use of these cationic dendrimers is hampered by their known toxicity in-vitro and in-vivo (Brazeau et al 1998; Malik et al 2000; El-Sayed et al 2002; Jevprasesphant et al 2003a, b). Before development for potential use in many biomedical applications, the biocompatibility of cationic dendrimers must be fully understood. However, little is known of their possible mechanisms of cell death. This information is vital for the development of safe clinical applications. To date, only one research group has shown that the cytotoxicity induced by PAMAM dendrimers in hepatocytes was characterized by apoptosis (Kawase et al 2001; Higashiyama et al 2003). In the present study, we found that starburst PAMAM and DAB dendrimers could cause apoptosis in cultured macrophage cells. Nuclear staining (Figure 2), DNA fragmentation assay (Figure 3) and flow cytometry analysis (Table 1) confirmed the apoptotic effects of cationic dendrimers. Starburst PAMAM and DAB dendrimers exerted toxic effects on the proliferation of macrophage cells in a typically dose-dependent manner (Figure 1), consistent with previous studies (El-Sayed et al 2002; Jevprasesphant et al 2003a, b). Other studies have found that the haemolytic and cytotoxic effects of a broad range of dendrimers are most dependent on their type and number of surface groups (Malik et al 2000; El-Sayed et al 2002; Jevprasesphant et al 2003a, b).

Morgan et al (1988, 1989) demonstrated that cytotoxicity induced by polycations was related to the number of attachment sites to which polycations bind and the distribution of anionic domains on the cell surface. Polycations interacted with membrane proteins and phospholipids, leading to disturbed membrane function and structure (Morgan et al 1989). Malik et al (2000) observed that the membrane interactions of adsorbed dendrimers caused red blood cell haemolysis. Cytotoxic effects may also be mediated by cellular uptake and subsequent activation of signal transduction pathways (Regnstrom et al 2003). Hong et al (2004) demonstrated that the uptake of PAMAM dendrimer into cells was related to the formation of holes in the membrane. However, Morgan et al (1989) demonstrated that internalization of polylysine was not a prerequisite for cytotoxicity as inhibition of microtubules and microfilament formation was not effected. The mechanism of cell death induced by polycations is not yet fully understood and the literature is inconclusive. For example, Fischer et al (2003) reported that poly(ethylenimine) exerted a necrotic type of cell death by the early and rapid loss of plasma membrane integrity in L929 mouse fibroblasts. However, Regnstrom et al (2003) found that poly(ethylenimine) activated genes involved in apoptosis, oncogenesis, differentiation and cell cycle regulation. Since necrosis and apoptosis can both co-exist in a system, necrotic cell death might also be in part responsible for the cell viability decrease in RAW 264.7 cells, indicated by the release of 40% of LDH upon addition of 10 $\mu g\,mL^{-1}$ cationic dendrimers (Figure 1). This indicates that there are intermediate forms of cell death that cannot completely relate to one or another. Furthermore, apoptosis also depends on the type of cell line used. We also found that macrophage cells were sensitive to induction of apoptosis by dendrimers (PAMAM and DAB), whereas NIH/3T3 cells (mouse fibroblast) and BNL CL.2 (mouse liver) cells did not undergo apoptosis (data not shown). This discrepancy may be contributed to by the different surface receptors for activation of signalling pathways within the cells (Nohe & Petersen 2004).

The extent of cytotoxicity induced by polycations also depends on various parameters such as size, conformation, molecular weight and cationic charge density of polycations (Morgan et al 1989; Fischer et al 2003). In a previous report, PAMAM dendrimers with a globular structure were found to be less cytotoxic than polycations with a linear structure (Fischer et al 2003). As the geometry of dendrimers is similar to cationic liposomes, our findings regarding induction of apoptosis by dendrimers in RAW 264.7 macrophages were consistent with a previous study on cationic liposomes (Takano et al 2003). The involvement of reactive oxygen species in macrophage apoptosis by cationic liposomes has been confirmed (Aramaki et al 1999). In Table 1, DAB dendrimer (1.9 nm, 64 surface NH_2 groups) was more apoptotic to RAW 264.7 than PAMAM dendrimer (4.3 nm, 128 surface NH_2 groups). This may be attributed to structural features such as geometry and the degree of ionization of amine groups.

Our study also revealed that apoptosis induced by cationic dendrimers can be partially blocked by the general caspase (cysteine proteases) inhibitor zVAD-fmk, as shown in Table 1. This indicated that apoptosis in macrophage cells involved a caspase-dependent pathway induced by cationic dendrimers. zVAD-fmk can irreversibly bind to the catalytic site of caspase proteases and prevent caspase-activated DNase from nicking the DNA in cultured cells (Jacobsen et al 1996). Because of the complexity of the apoptosis signal pathway, we used a broad-spectrum caspase inhibitor to study the prevention of apoptosis induced by dendrimers. Other caspase inhibitors need to be investigated. Finally, since a localized inflammatory response in macrophages has been observed in necrotic tissue in-vivo, the introduction of apoptosis induced by dendrimer into macrophages may inhibit or regulate the inflammatory process.

Conclusions

Cationic dendrimers were cytotoxic to RAW 264.7 cells and apoptosis occurred. Apoptosis induced by dendrimers also depends on the type of cell line used. In addition, our study revealed that apoptosis induced by cationic dendrimers can be partially blocked by the general caspase inhibitor- zVAD-fmk, suggesting a caspase-dependent pathway of apoptosis. These results may be helpful for optimizing the biocompatibility of dendrimer used for targeted drug/gene delivery.

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