Mechanistic studies of *in vitro* cytotoxicity of PAMAM dendrimers in mammalian cells

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ABSTRACT

The *in vitro* cytotoxic response of human dermal and colon cell lines to structurally well defined full generation cationic dendritic polyamidoamine (PAMAM) nanoparticles was investigated. Dendrimers of generations G4, G5, G6 were chosen for this study. PAMAM dendrimers have been demonstrated to elicit a well defined cytotoxicological response from Alamar Blue, Neutral Red and MTT assays, where the response increases systematically with dendrimer generation and number of surface amino groups. A good correlation was found between the EC_{50} values of these assays. This systematic response is furthermore demonstrated for the generation of reactive oxygen species, mitochondrial membrane potential, inflammatory responses, lysosomal activity, cell cycle interference, caspase activation, onset of apoptosis and levels of DNA damage. The mechanism of endosomal escape of PAMAM by the so-called 'proton-sponge effect' was also studied. The results are consistent with a pathway of the endosomal uptake of PAMAM, followed by the endosomal rupture and subsequent localisation of PAMAM dendrimers in the mitochondria, leading to PAMAM generation, dose and time dependant biphasic ROS production, mitochondrial membrane potential decay and caspase- 8 and 3 activation, inflammatory responses (TNF-α, IL-6 and IL-8 expression), apoptosis and DNA damage (by TUNEL assay). Overall, significant differences are observed between the responses of the dermal and colon cell lines, and it is suggested that these can be understood in terms of differing intrinsic antioxidant levels.

Keywords: ROS, lysosomal activity, ROS localization in mitochondria, EC50 corelation, Apoptosis.

1 INTRODUCTION

Poly(amidoamine) (PAMAM) dendrimers are the first complete dendrimer family to be synthesized, characterized and commercialized (Esfand and Tomalia, 2001) and are recognized as a unique new class of synthetic nanostructures. Dendrimers allow the precise control of size, shape and placement of functional groups that is advantageous for many life science applications. Their systematically variable structural architecture and large internal free volume make these dendrimers an unique class of molecule for various biomedical applications including drug (Na et al, 2006), DNA (Guillot-Nieckowski et al., 2007,), and siRNA (Zhou et al., 2006) delivery, and as MRI probes (Swanson et al. 2008) etc.

PAMAM dendrimers contain a 2-carbon ethylenediamine core on which the terminal amidoamines are attached yielding a highly branched radial structure having tertiary amine branches and primary amino groups on the surface. With the successive generations (G0-G10) the diameter and the number of surface amino groups systematically increase (http://www.dendritech.com/pamam.html).

It is reported however that PAMAM dendrimers induce oxidative stress by producing reactive oxygen species (ROS) (Mukherjee et al., 2010b, Lee et al., 2009) and can lead to a cytotoxic response (Mukherjee et al., 2010a; Naha et al., 2009). Although the response is mild, the molecular definition and the systematic variability of the size and structure of the PAMAM dendrimers render them an ideal material in which to study cytotoxic responses and elucidate their mechanisms. In both mammalian (Mukherjee et al., 2010a, 2010b) and ecotoxicological (Naha et al., 2009) studies, clear structure – activity relationships have been demonstrated, indicating that the toxicity increases in proportion to the surface area or number of surface groups.

In this proceeding paper, the mechanism of PAMAM toxicity is demonstrated in terms of generation dependant ROS production, cellular lysosomal activity, apoptosis and DNA damage. Relationships between the responses of the standard cytotoxicity assays- MTT, Alamar Blue (AB) and Neutral Red (NR) assays are also elucidated and further framed within the context of ROS production, lysosomal activity, apoptosis and DNA damage.

2 MATERIALS AND METHODS

2.1 Test Materials

Polyamidoamine (PAMAM) dendrimers, G4, G5 and G6, were purchased from Sigma Aldrich Ltd. (Ireland). All the particles have an ethylenediamine core and PAMAM G4, G5 and G6 have respectively 64, 128 and 256 functional primary amino groups on the surface. The nominal diameters of the PAMAM G4, G5 and G6 dendrimers are 4.5, 5.4 and 6.7 nm respectively (http://www.dendritech.com/index.html).

2.2 Particle characterization

Size and zeta potential of PAMAM dendrimers were measured in dH2O, PBS, and in cell culture media as reported previously (Mukherjee et al., 2010a). It's interaction with the media was also studied spectrophomoterically (Mukherjee et al., 2010a).

2.3 Alamar Blue (AB), Neutral Red (NR) and MTT assays

AB, NR and MTT assays were performed in 96 microwell plate format following the standard procedure as have been reported previously (Mukherjee 2010a).

2.4 ROS study

Carboxy-DCFDA dye was used to measure the ROS generated upon different generations of PAMAM exposure. This assay was performed in the black 96 microwell plate format following the standard procedure as published previously (Mukherjee et al., 2010b). The intracellular localization of ROS was also studied by confocal microscopy in glass bottomed plate format using double carboxy-H2DCFDA dye and Mitotracker red dye of Invitrogen, Ireland (Mukherjee et al., 2010b).

2.5 Lysosomal and mitochondrial activity study

Lysosensor, Lysotracker and Mitotracker dye of Invitrogen (Ireland) was used for this study. TECAN GENios (Grodig, Austria) plate reader was used for the quantitaive measurement of lysosomal activity using 96 microwell plate format and the qualitative estimation was performed in confocal microscope . The detailed procedure was published in Mukherjee et al., 2010b.

2.6 Apoptosis study

The apoptosis study was performed on HaCaT cells following the satndard protocol as reported in Mukherjee et al., 2010b. YO-PRO-1/Propidium iodide dyes were used to analyse the apoptotic cell population upon 24 h PAMAM exposure. The cells were then analysed in the Partec Flow Cytometer (Partec UK Limited, U.K.).

2.7 TUNEL assay

The TUNEL assay was performed to assess the DNA damage upon PAMAM exposure in HaCaT cells. Following the 24h PAMAM exposure the assay was performed using APO-DIRECT^{TM} KIT (BD Pharmingen^{TM}, U.K.) protocol and were analysed in the Partec Flow Cytometer (Partec UK Limited, U.K.).

3 RESULTS

3.1 AB, NR and MTT assays

A generation dependent toxic response of PAMAM dendrimers, whereby the toxicity increases with increasing generation (Mukherjee et al., 2010a). In the 24 h exposure study, the MTT assay was found to be most sensitive, the NR assay the least. In general, SW480 cells were seen to exhibit a higher sensitivity to the dendrimers than the HaCaT cells, however, using the NR assay, at lower concentrations of all three PAMAM generations, an apparent stimulatory response was found in HaCaT cells but was not as prominent in SW480 cells (Mukherjee et al., 2010b). In HaCaT cells, this apparent stimulatory response is similarly generation dependent, G6 showing the highest stimulatory response and G4 the lowest. The results also suggest that the concentration at which the highest stimulatory response is observed for each dendrimer is directly correlated with the EC_{50} concentration found in the MTT assay and that the toxic/non-toxic transition concentration (or threshold concentration) found in the NR assay correlates well with the EC_{50} concentration found in AB assay (Figure 1, Mukherjee et al., 2010b). Such a correlation is observed for all 3 dendrimer generations suggesting origin in the underlying response mechanisms and thus the toxic pathway in HaCaT cells was investigated further in this study

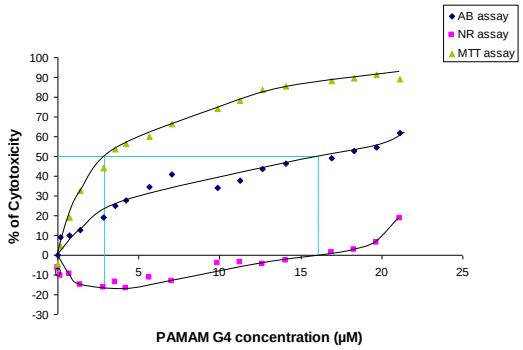

Figure 1. Correlation of EC_{50} concentrations from MTT and AB assay with the dose response found in NR assay upon PAMAM G4 exposure to HaCaT cells (reproduced from Mukherjee et al., 2010b).

3.2 Lysosomal and mitochondrial activity study

At concentrations below the EC_{50} concentrations found in the MTT assay, an increased lysosomal activity compared to unexposed negative control cells was observed in HaCaT cells after 24 h exposure to all three dendrimer generations, but notably this activity was not observed within the first 6 h of dendrimer exposure where the lysosomal activity was found to be lower than the control

(Mukherjee et al., 2010b). The increase in lysosomal activity is also dependant on the generation of PAMAM, whereby G6 showed highest lysosomal activity and G4 the lowest. Such significant lysosomal stimulatory activity was not observed after 24 h exposure of PAMAM in the lysotracker assay in SW480 cells. However, a small increase was observed between 1.5 µM to 1.87 µM of PAMAM G6 concentration after 6 h exposure. The Mitotracker study using confocal microscopy showed a decrease in mitotracker staining with increasing PAMAM concentration and followed the trend observed in the MTT assay.

3.3 ROS study

The ROS study suggests that, in both HaCaT and SW480 cells, ROS is produced in a biphasic way (Figure 2, Mukherjee et al., 2010b). An initial increase in ROS levels was observed during the first hours but this decreased after ~ 4 h exposure in HaCaT cells and after 1 h exposure in SW480 cells. A later rise in ROS levels was observed after 24 h exposure in HaCaT cells, whereas this intracellular ROS increase was observed after 2 h of PAMAM exposure in SW480 cells.

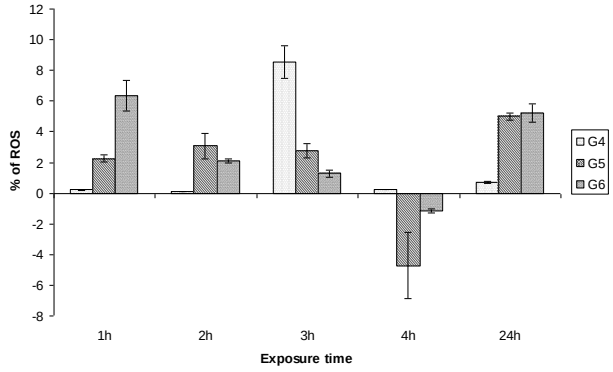

Figure 2. Reactive Oxygen Species study after different time point exposure of 1µM PAMAM G4, G5 and G6 dendrimers- a) in HaCaT cells, b) in SW480 cells (reproduced from Mukherjee et al., 2010b).

The initial ROS was found in some sacs/vessicles, while the late ROS was co-localized in the mitochondria (Figure 3, Mukherjee ta al., 2010b).

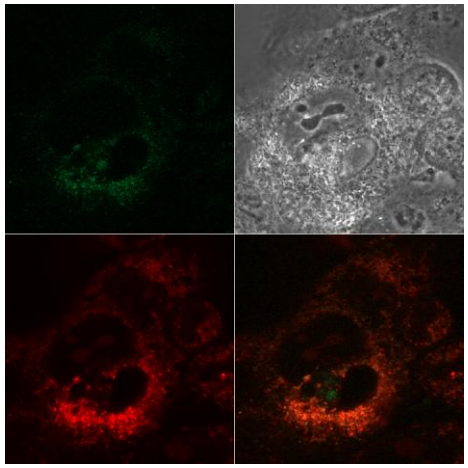

Figure 3. Localization of ROS in the mitochondria of HaCaT cells after 24 h exposure of 1µM PAMAM G6- Top Left: only Carboxy H_2DCFDA staining, Top Right: only Mitotracker staining, Bottom Left: overlay Carboxy H_2DCFDA and Mitotracker staining, Bottom Right: phase contrast image (reproduced from Mukherjee et al., 2010b).

3.4 Apoptosis study

The apoptosis study of HaCaT cells exposed to G4, G5 and G6 dendrimers suggest that with increasing concentration, the percentage of healthy and early apoptotic cells decreases, whereas the late apoptotic and dead cell populations increases. It is notable that the decay of the healthy population is monotonic from the lowest concentrations. It was also observed that with increasing generation of PAMAM, cells enter apoptosis at a lower dose than the previous generation.

3.5 TUNEL assay

The TUNEL assay data further demonstrates a generation dependent toxicity. At a 3.17 µM PAMAM exposure of HaCaT cells, G4, G5 and G6 showed 4.69%, 25.87% and 89.63% DNA breakage respectively (Mukherjee et al., 2010b). The percentage damage at a fixed concentration is seen to be almost linearly correlated with the number of surface amino groups per generation, and therefore with the molar concentration of amino groups.

4 DISCUSSION AND CONCLUSSION

The DLS study have shown the increase in the size of the dendrimers upon dispersion in the cell culture media, while the zeta potential also decreases upon its dispersion (Mukherjee et al., 2010a). It suggest the interaction of PAMAM with the cekll culture media, which was also confirmed from the spectroscipic study of PAMAM-media interaction (Mukherjee et al., 2010a).

The results of the cytotoxicity study have shown the toxicity of the PAMAM dendrimers was found to increase

with increasing dendrimer generation, and therefore number of surface amine groups, for both HaCaT and SW480 cells and all end points (Mukherjee et al., 2010a).

Notably, a generation dependant stimulatory dose response was observed in the NR assay in HaCaT cells (Mukherjee et al., 2010b). With increasing generation and therefore diameter, zeta potential and number of surface amino groups of the dendrimers, the maximum stimulatory response increases in the order G4(16%) < G5(17%) < G6(25%), although the dose of the maximum response and the toxic/non-toxic transition dose decreases with increasing generation (Figure 1). It was also observed that the maximum stimulatory activity found in the NR assay correlates well with the EC_{50} of the MTT assay, whereas the toxic/non-toxic threshold concentration found in the NR assay correlates well with the EC_{50} found in the AB assay (Figure 1) for all generations. Such a stimulatory response was not as pronounced in the response of the NR assay in SW480 cells. Given the systematic nature of the observed responses, a further examination may lead to a greater understanding of the underlying mechanisms.

A commonly accepted paradigm for nanoparticle cytotoxicity is one of endocytosis, encapsulation in endosomes and then lysosomes, and the increased lysosomal activity observed at low doses could point towards such a mechanism. However, such a mechanism is reported primarily for anionic particles, and cationic particles have been shown to localise in mitochondria and produce a cytotoxic response via the mitochondrial injury pathway (Xia et al 2008 and 2006), generating ROS as a result (Xia et al., 2006; Lee et al., 2009).

In the current study, the lysosomal activity was observed to be less than control at the early stages up to 6 h. The absence of lysosmal activity at this stage suggests that, potentially after early endosomal transport, the dendrimers are released into the cytosol where they are free to interact with intracellular membrane structures. PAMAM dendrimers of higher generations are proposed to be good DNA, siRNA transfection agents, through a mechanism of entry through the endosomal pathway and release into the cytoplasm by the proton sponge hypothesis, thereby avoiding lysosomal degradation (Guillot-Nieckowski et al., 2007; Zhou et al., 2006). Although PAMAM can also enter the cytosol via other possible ways, e.g., through nano-hole formation in the cell membrane (Hong et al., 2006).

The intracellular ROS generation study has suggested that in HaCaT cells, ROS is generated in a biphasic way upon PAMAM exposure. Maximum initial ROS was generated in the first few hours of PAMAM exposure and the late ROS was found after 24 h exposure. Although, early ROS are distributed throughout the cytosol, within 24 h they are localised in the mitochondria as shown in Figure 3. This is consistent with the observation by Lee et al, 2009, of the co-localisation of dendrimers in mitochondria of human lung cells (WI-26 VA4) after 16 h exposure. Subsequent mirochondrial rupture can result in the late increase in lysosomal activity and trafficking of dendrimers in these vesicles.

The results reported here are therefore consistent with a pathway of early endosomal transport of PAMAM dendrimers into the cytosol, followed by localisation in the mitochondria (Lee et al., 2009) leading to ROS production and disruption of the mitochondrial electron transduction chain, and additional O_2^- production (Donaldson et al., 2005) resulting in oxidative stress, and apoptosis and DNA damage (Mukherjee et al., 2010b).

The toxic pathway of the cationic PAMAM dendrimers is confirmed as localisation in the mitochondria leading to ROS production and resulting in oxidative stress, apoptosis and DNA damage. Beginning with ROS production, all processes are systematically generation dependent, further illustrating the importance of such structurally controlled and defined species for the study of the underlying toxic mechanisms. The differences observed between the different cytotoxicity assays can be understood in terms of the effect of the sequence of events on the cellular mechanisms. It is suggested that the differences in the responses of the two cell lines is based on differences in intrinsic levels of anti-oxidants, the first line of defence against the toxic pathway.

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