行政院國家科學委員會專題研究計畫　期中進度報告

以抗體-陽離子性樹枝型高分子載體為目標導向基因傳送之研究(1/3)

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行政院國家科學委員會補助專題研究計畫成果報告

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※ **以抗體-陽離子性樹枝型高分子載體為目標導向基因傳送之研究(1/3)** ※

※ **Targeted gene delivery using antibody-conjugated cationic dendrimer** ※

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計畫參與人員：邱鉉文,黃永成

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一.中文計畫摘要:

對於有關陽離子樹枝體對細胞死亡機轉的探討文獻是比較少的，而這些成分對於生物相容性以及未來若用人體應用中這些資訊是不可不知的。本論文實驗分為陽離子樹枝體不同的系統的載體對於老鼠巨噬細胞(RAW264.7)細胞死亡機轉做一個探討。我們選擇一般較為常用的 PAMAM 和 DAB 兩種陽離子樹枝體對老鼠巨噬細胞死亡影響，陽離子樹枝體添加劑量和細胞的存活率有呈現典型 dose-dependent，並且也會誘發細胞凋亡的產生。流式細胞儀的分析結果中，Sub-G1 分佈的亞二倍體隨著陽離子樹枝體劑量的增加而分佈區域也有增加。並且從 Sub-G1 分佈得知 DAB 相對於相同劑量下 PAMAM 所誘發細胞凋亡的程度較為嚴重，此外同樣的利用 caspase inhibitor (zVAD-fmk)對細胞凋亡路徑做一個研究。結果顯示經 caspase inhibitor (zVAD-fmk) 處理後會抑制細胞凋亡部分，所以陽離子樹枝體所造成細胞凋亡的路徑可能為 caspase-dependent pathway。此外還有再用將陽離子樹枝體對於其他細胞（細胞株為老鼠母纖維細胞(NIH/3T3)和老鼠肝細胞(BNL CL.2)）做一個測試，其結果並沒有發生細胞凋亡的現象。

二.英文計畫摘要:

Cationic dendrimers possess attractive nano-sized architectures and these characteristics make them suitable as targeted drug/gene delivery systems. However, very little is known about their possible cytotoxic mechanisms in cellular system. Also, this information is vital for the future development of safe biomedical systems. The apoptotic and necrotic effects of starburst polyamidoamine (PAMAM) and polypropylenimine (DAB) dendrimers in cultured RAW 264.7 murine macrophage-like cells were investigeted. 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 by cationic dendrimers. Analysis from flow cytometry demonstrated an increase of hypodiploid DNA population (sub-G1) and a simultaneous decrease of diploid DNA content, indicating that DNA cleavage occurred after exposure of the cells with 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 pretreatment of RAW 264.7 cells with the general caspase inhibitor (zVAD-fmk) did prevent some degree of apoptosis induced by cationic dendrimers, suggesting that apoptosis in macrophage cells involved a caspase-dependent pathway. We also found that macrophage cells were 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 data could be helpful for optimizing the biocompatibility of dendrimers used for targeted drug/gene delivery.

三.計畫緣由與目的:

Cationic dendrimers are a family of highly branched polymers characterized by their unique structure and properties (Boas & Heegaard 2004; Florence & Hussain 2001; Tomalia 1995). These attractive nano-sized architectures of cationic dendrimers make them suitable

in many pharmaceutical applications such as delivery of DNA and oligonucleotides into eukaryotic cells, solubilization of spraringly soluble drugs, nanocarries for transepithelial transport, and platforms for cancer therapeutics (Cloninger 2002; Dennig & Duncan 2002; Esfand & Tomalia 2001; Haensler & Szoka 1993; Pistolis et al 1999; 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; Maksimenko et al 2003; Zinselmeyer et al 2002). 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 (Choksakulnimitr et al 1995; Fischer et al 2003; Morgan et al 1989). 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 (generation 3 and 5) did not exhibit *in vivo* cytotoxicity in mice (Roberts et al 1996). Very little is known of their possible cytotoxic mechanisms thereof.

There are two major mechanisms by which cells die in various biological systems: necrosis and apoptosis (Majno & Joris 1995). Necrosis is related with an inflammatory and a degenerative process. Cells undergoing necrosis characteristically illustrate mitochondrial swelling, lose membrane integrity, turn off metabolism, and release of cytoplasmic component that stimulates 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 (Cummings et al 1997; Kerr et al 1972). To date, only one research group shows that the cytotoxicity induced by PAMAM dendrimers in hepatocytes was characterized by apoptosis (Higashiyama et al 2003; Kawase et al 2001). Therefore, better understanding of the mechanisms of cell death induced by cationic dendrimers will present benefits to further the development of appropriate delivery systems.

Dendrimers are primarily captured by the mononuclear phagocyte system (MPS) 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, 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 this study, RAW 264.7, murine macrophage-like cells, was selected as a “model cell line” to represent the physiological scavengers of foreign nanoparticles in the body.

The aim of this 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 dendrimer used for targeted drug/gene delivery.

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四.結果與討論:

Cell viability assays

Figure 1A illustrates that cell viability was dramatically reduced with increasing concentrations of PAMAM and DAB dendrimers. Also, cationic dendrimers own very low IC_{50} (< 10μg/mL (Figure 1A)) indicating macrophages are very toxically sensitive to the exposure of cationic dendrimers. In the previous study, macrophages demonstrated the higher toxic sensitivity than other cultured cells tested, which are consistent with our findings (Choksakulnimitr et al 1995). As for the LDH release assays (Figure 1B), similar results were observed in dendrimer-treated cells showing 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).

Morphology changes of RAW 264.7 cells induced by dendrimer treatment

The hallmarks of apoptosis such as chromatin condensation and cell shrinkage developed upon 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 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 15μg/mL, the relative brightness of DNA ladders also increased. From these observations, PAMAM and DAB dendrimers induced apoptosis in RAW264.7 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 of PAMAM and DAB dendrimers showed an increase of hypodiploid DNA peak and a simultaneous decrease of diploid DNA content, indicating that DNA cleavage occurred after exposure of the cells with cationic dendrimers (Table 1). Also, cells treated with DAB dendrimer induced a higher percentage of sub-G1 population than those treated with PAMAM dendrimer at the same dose. This indicated that DAB dendrimer induced more apoptotic cells than PAMAM dendrimer.

zVAD-fmk inhibition

As seen by flow cytometry in Table 1, PI-stained cells after treatment of zVAD-fmk followed by adding PAMAM

and DAB dendrimers demonstrated the increase of diploid DNA content and decrease of sub-G1 peak with cells treated with zVAD-fmk. This indicates that apoptosis induced by cationic dendrimers can be inhibited in some degree by the pretreatment of zVAD-fmk, suggesting a caspase-dependent pathway of apoptosis may be involved. When the concentration of zVAD-fmk was increased up 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

Like cationic liposomes and other cationic carriers, dendrimers may be the potential candidates used as vaccine adjuvants to evoke subsequent humoral and cellular immunity (Gregoriadis et al 1997; Regnstrom et al 2003; Singh et al 2000). However, the use of these cationic dendrimers is hampered by their well-known toxicity *in vivo* and *in vitro* (Brazeau et al 1998; El-Sayed et al 2002; Jevprasesphant et al 2003a, b; Malik et al 2000). Before developing of 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 future development of safe clinical applications. To date, only one research group shows that the cytotoxicity induced by PAMAM dendrimers in hepatocytes was characterized by apoptosis (Higashiyama et al 2003; Kawase et al 2001). 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 effect 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 found that the hemolytic and cytotoxic effects of a broad range of dendrimers was most dependent on their type and number of surface groups (El-Sayed et al 2002; Jevprasesphant et al 2003a, b; Malik et al 2000).

Existing literature 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 (Morgan et al 1988; Morgan et al 1989). Polycations interacted with membrane proteins and phospholipids, leading to disturb membrane function and structure (Morgan et al 1989). Malik et al. observed that the membrane interactions of adsorbed dendrimers caused red blood cell hemolysis (Malik et al 2000). Cytotoxic effect may also be mediated by cellular uptake and subsequent activation of signal transduction pathways (Regnstrom et al 2003). Previous study also demonstrated that the uptake of PAMAM dendrimer into cells was related to hole formation in membrane (Hong et al 2004) However, Morgan et al. demonstrated that internalization of polylysine was not a prerequisite for cytotoxicity ,as inhibition of microtubules and microfilament formation was not effected (Morgan et al 1989). The responsible mechanism of cell death induced by polycations is not yet fully understood and discussed inconclusively in the literature. For example, Fischer et al. reported that

Poly(ethylenimine) (PEI) exerted a necrotic type of cell death by the early and rapid loss of plasma membrane integrity in L929 mouse fibroblasts (Fischer et al 2003). However, in a recent publication it was found that PEI activated genes involved in apoptosis, oncogenesis, differentiation, and cell cycle regulation (Regnstrom et al 2003). Since necrosis and apoptosis both co-existed in a system, necrotic cell death might also be in part responsible for cell viability decrease in RAW 264.7, indicated by the release of 40% of LDH upon addition of 10μg/mL cationic dendrimers (Figure 1) . It 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 lines 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 was not shown). This discrepancy may be contributed by the differences of surface receptors for activation of signaling pathways within the cell (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). Since dendrimer's geometry was similar to cationic liposomes, our findings resulting from induction of apoptosis by dendrimers in RAW 264.7 macrophages were consistent with previous study of cationic liposomes (Takano et al 2003). The involvement of reactive oxygen species (ROS) in macrophage apoptosis by cationic liposomes has been confirmed (Aramaki 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 apoptosis induced by cationic dendrimers can be partially blocked by the general caspase (cysteine proteases) inhibitor- zVAD-fmk, as shown in Table 1. This result 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 apoptosis-signal pathway, we used the broad spectrum caspase inhibitor to study the prevention of apoptosis induced by dendrimers. Additional studies using other caspase inhibitors will be investigated in the future. 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 inflammatory process.

五.計劃成果自評:

本實驗結果已發表於 SCI 學術期刊:

Kuo, J. H., Jan, M.S., Chiu, H. W., "Mechanism of cell death induced by cationic dendrimers in RAW 264.7 murine macrophage-like cells," *Journal of Pharmacy and Pharmacology*, Vol. 57, No. 4, 2005, pp. 489-496. (SCI)

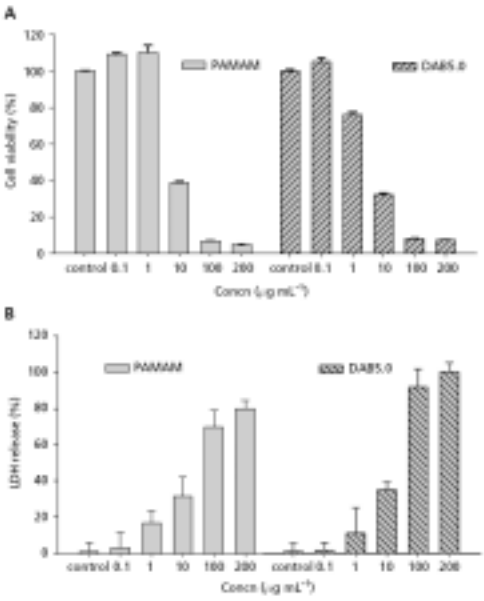

Figure 1 A. Cytotoxicity assays measuring generated dehydrogenases. Positive control cells were grown without adding cationic dendrimers. Results are reported as % cell viability (average optical density/average positive control optical density) ± s.d., n = 6. B. Lactate dehydrogenase (LDH) release. 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.

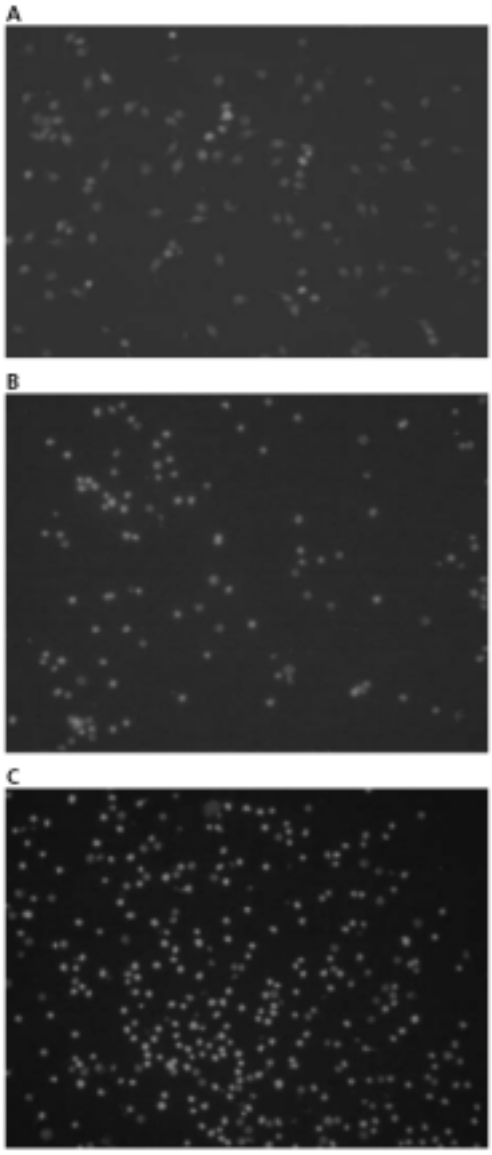

Figure 2 Morphology changes of RAW 264.7 cells induced by cationic dendrimer treatment for 24 h. Untreated cells (A) were compared with cells treated with 10 μg mL^{-1} PAMAM dendrimer (B) and 10 μg mL^{-1} DAB dendrimer (C) (200×).

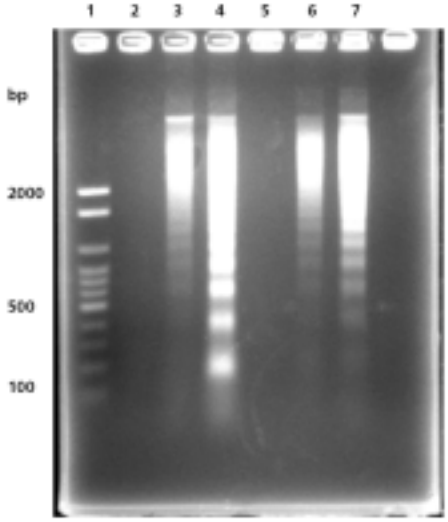

Figure 3 Agarose gel electrophoresis of DNA extracted from RAW 264.7 cells treated with cationic dendrimers for 24h. Lane 1: DNA marker; lane 2: control (untreated cells); lane 3: 10 $\mu g\,mL^{-1}$ PAMAM dendrimer treatment; lane 4: 15 $\mu g\,mL^{-1}$ PAMAM dendrimer treatment; lane 5: control (untreated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

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

	[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.