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Changes in apoptosis-related gene expression and cytokine release in breast cancer cells treated with CpG-loaded magnetic PAMAM nanoparticles

Negar Taghavi Pourianazar[a,*], Ufuk Gunduz[a,b]

[a] Middle East Technical University, Department of Biotechnology, 06800, Ankara, Turkey
[b] Middle East Technical University, Department of Biological Sciences, 06800, Ankara, Turkey

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ABSTRACT

CpG-oligodeoxynucleotide (CpG-ODN) can function as an immune adjuvant. Previously, we showed that stimulation of breast cancer cells with CpG-ODN conjugated with PAMAM dendrimer-coated magnetic nanoparticles (DcMNPs) has induced apoptosis. The aim of the current study was to evaluate the expression levels of some apoptosis-regulating genes in several human breast cancer cells treated with CpG/DcMNPs. Treated MDA-MB231 cells showed an increase in *Noxa* and *Bax* gene expression levels, whereas the expression level of *Survivin* decreased. Similarly, *Noxa* gene was overexpressed in treated MCF7 cells. In treated SKBR3 cells, a decline in the *c-Flip* mRNA level was determined. Furthermore, release of cytokines, IL-6, IL-10, and TNF-α, was determined in cell culture supernatants. CpG/DcMNP treatment leads to an increase in the release of IL-6 in MDA-MB231 and SKBR3 cells, whereas release of IL-10 and TNF-α did not change significantly. It is indicated that CpG-ODN may show its cytotoxic effect by regulating the expression of apoptosis-related genes and the release of cytokine in breast cancer cells.
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1. Introduction

Toll-like receptors (TLRs) are type I transmembrane proteins that recognize a set of conserved molecular structures called pathogen-associated molecular patterns (PAMPs) (Zhang et al., 2013). TLRs are broadly distributed in various cell types of the immune system. However, the expression of TLR9 has been detected in different normal epithelial and cancer cells, including breast, brain, lung, gastric, and prostate cancers (O'Neill et al., 2013).

TLR9 which is located intracellularly in endosomes and endoplasmic reticulum is essential for recognition of microbial CpG DNA or synthetic unmethylated CpG oligodeoxynucleotide (CpG-ODN) (Cao et al., 2013). The binding of CpG DNA to TLR9 and the subsequent endosomal maturation are thought to activate the TLR9-mediated MyD88-dependent NF-κB signaling pathway (Zhang et al., 2014). Studies have shown that both bacterial CpG motifs and synthetic oligodeoxynucleotides (ODNs) act as PAMPs to activate TLR9-positive cells (Liu et al., 2003). We have previously investigated the apoptosis in breast cancer cells after treating with CpG-loaded dendrimer-coated magnetic nanoparticles (DcMNPs). These nanoparticles offer the potential to achieve selective and efficient delivery of CpG-ODN by using external magnetic fields. Magnetic nanoparticles (MNPs) based on iron oxide as drug delivery system carrying the anticancer agent can be targeted to the tumor site, and accumulated in cancer cells by the help of an applied magnetic field. However, such nanoparticles need some surface modification to protect them from reticuloendothelial system and increase their stability *in vivo*. Poly(amidoamine) (PAMAM) dendrimers having nanometer size and manageable molecular weight, biocompatibility, and non-immunogenicity are suitable options for stabilizing the magnetic nanoparticles while

Abbreviations: APTS, aminopropyltriethoxysilane (also known as Aminosilane); BSA, bovine serum albumin; CpG-ODN, CpG oligodeoxynucleotide; C_T, threshold cycle; DcMNPs, PAMAM dendrimer-coated magnetic nanoparticles; ELISA, Enzyme-Linked ImmunoSorbent Assay; ER^-, estrogen receptor-negative; EtBr, ethidium bromide; G_0, Generation 0; IL, interleukin; MNPs, magnetic nanoparticles; PAMAM dendrimer, poly(amidoamine) dendrimer; PAMPs, pathogen-associated molecular patterns; pDCs, plasmacytoid dendritic cells; PI, propidium iodide; qRT-PCR, quantitative real-time polymerase chain reaction; TLR9, toll-like receptor 9; TLRs, toll-like receptors; TNF-α, tumor necrosis factor-α; w/w ratio, weight per weight ratio.

* Corresponding author at: Department of Biological Sciences, Middle East Technical University, Ankara, 06800, Turkey.
E-mail address: Negar_taghavi22@yahoo.com (N. Taghavi Pourianazar).

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providing them the bioavailability (Taghavi Pourianazar et al., 2014; Mishra et al., 2011). CpG/DcMNP conjugates could provide an apoptotic signal in a time- and concentration-dependent manner for breast cancer cells *in vitro* (Taghavi Pourianazar and Gunduz, 2016).

Apoptosis is one of the major mechanisms of cell death in response to cancer therapies (Tsujimoto, 1998). Shifting the balance between pro- and anti-apoptotic proteins may induce apoptosis or survival in malignant cells (Thomadaki and Scorilas, 2008; Daidone et al., 1999). The dynamic interplay between pro-death proteins such as Bax, Noxa, and Puma and pro-survival proteins such as Bcl-2, C-Flip, and Survivin control programmed cell death (Abedin et al., 2007; Kang and Reynolds, 2009; Seo et al., 2011; Zapata et al., 1998).

In this study, the expression patterns of some anti-apoptotic (*Bcl-2, C-Flip*, and *Survivin*) and pro-apoptotic (*Bax, Noxa*, and *Puma*) genes were analyzed in CpG/DcMNP-treated breast cancer cell lines. This will provide the scientific rationale for CpG/DcMNP conjugate as a possible therapeutic for breast cancer treatment.

CpG-ODN which is considered as an immune adjuvant interacts with TLR9 (Démoulins et al., 2014). This interaction leads to the secretion of large amounts of proinflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-α (TNF-α), IL-12, and up-regulation of costimulatory molecules which could mediate anti-tumor effects on cancer cells (Liu et al., 2003; Liang et al., 2013; Lim et al., 2010). Thus, interactions between CpG DNA and TLR9 effectively bridge innate and acquired immunities (Tanaka et al., 2010). In this research, to examine the changes in release of cytokines, IL-6, IL-10, and TNF-α, we analyzed the influence of CpG conjugated with DcMNPs on cytokine release in MDA-MB-231, SKBR3, and MCF7 breast cancer cell lines.

2. Materials and methods

2.1. Synthesis of PAMAM dendrimer-coated magnetic nanoparticles

The synthesis of MNPs, aminosilane modification, and production of PAMAM-coated magnetic nanoparticles (DcMNPs) and their characterizations were executed (Taghavi Pourianazar and Gunduz, 2016). Briefly, for the synthesis of magnetic nanoparticles (MNP), co-precipitation method was used (Yakar et al., 2013). In order to have amine terminated groups, the synthesized magnetite was modified with aminosilane (APTS) and called "Generation 0" (G_0). Michael reaction was utilized to coat APTS-modified iron oxide nanoparticles with PAMAM dendrimer (Gupta and Gupta, 2005; Lan et al., 2007; Liu et al., 2006; Osaka et al., 2006; Pan et al., 2007).

2.2. CpG-ODN loading on G_7DcMNPs

CpG-ODN 2006 (K-ODN; also known as CpG-B) was mixed with G_7DcMNP solution (w/w ratio was 1:70), followed by gentle rotating at 37° C for 30 min to ensure the complex formation.

2.3. Tumor cells

MDA-MB-231 cell line was a kind gift from Prof. Dr. Ferit Avcu, Gülhane Military Medical Academy, Ankara. SKBR-3 cell line was from Rengül Çetin-Atalay, Middle East Technical University, Ankara and MCF7 cell line was purchased from ŞAP Institüte, Ankara. The cells (human breast cancer cell lines) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.2% gentamycin solution. Cells were incubated at 37 °C with 5% CO2 in a humidified incubator (Heraeus incubator, Germany).

2.4. Cytotoxicity of CpG/DcMNPs conjugates

The cytotoxic effect of CpG-ODN/DcMNP conjugate on MDA-MB231, SKBR3, and MCF7 cells was measured by XTT assay (Taghavi Pourianazar and Gunduz, 2016). Briefly, 8000 cells/well were seeded in 96-well plates and treated on the following day with free CpG, unloaded DcMNP, CpG-ODN/DcMNP complexes, or medium alone (control). After incubation of plates at 37 °C for 24, 48, and 72 h, XTT reagent and activator were added to each well, and analyzed on a Spectromax 340, 96-well plate reader (Molecular Devices, USA) (Roehm et al., 1991).

2.5. Flow cytometric analysis of cell apoptosis

In order to validate that the cells underwent apoptosis after treatment with CpG-ODN/DcMNP complexes, flow cytometry test was utilized (Taghavi Pourianazar and Gunduz, 2016). In brief, a total of 1×10^6 of MDA-MB231, SKBR3, and MCF7 cells were cultured and allowed to grow for 24 h. Then, cells were stimulated with free CpG-ODN, unloaded DcMNP, and CpG-ODN/DcMNP complexes for a period of 48 h. The cell death was tested with annexin V-fluorescein isothiocyanate apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, USA) in accordance with kit instructions. Stained cells with annexin V and propidium iodide (PI) solution were analyzed on a BD Accuri C6 flow cytometer (BD Biosciences) (Vermes et al., 1995).

2.6. RNA isolation and cDNA synthesis

MDA-MB231, SKBR3, and MCF7 cells grown in 6-well plate (2×10^5 cells/well) at 37 °C for 24 h were treated with 0.5 μg/ml

Table 1
Primers used in gene expression analyses.

Primer	Sequence (5′-3′)	Amplicon Size (bp)
Bax Sense	TCTGACGGCAACTTCAACTG	188
Bax Antisense	TTGAGGAGTCTCACCCAACC	
Noxa Sense	TGATATCCAAACTCTTCTGC	142
Noxa Antisense	ACCTTCACATTCCTCTCAA	
Bcl-2 Sense	TGTGGCCTTCTTTGAGTTC	166
Bcl-2 Antisense	CGGTTCAGGTACTCAGTCATC	
Survivin Sense	AGCCAGATGACGACCCCATAGAGG	60
Survivin Antisense	AAAGGAAAGCGCAACCGGACGA	
Puma Sense	GACGACCTCAACGCACAGTA	109
Puma Antisense	GTAAGGGCAGGAGTCCCAT	
C-Flip Sense	GAACATCCACAGAATAGACC	262
C-Flip Antisense	GTATCTCTCTTCAGGTATGC	
β-actin Sense	CCAACCGCGAGAAGATGA	97
β-actin Antisense	CCAGAGGCGTACAGGGATAG	

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