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Reversal of P-glycoprotein-mediated multidrug resistance by CD44 antibody-targeted nanocomplexes for short hairpin RNA-encoding plasmid DNA delivery

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ABSTRACT

Multidrug resistance (MDR) remains one of the major reasons for the reductions in efficacy of many chemotherapeutic agents in cancer therapy. As a classical MDR phenotype of human malignancies, the adenosine triphosphate binding cassette (ABC)-transporter P-glycoprotein (MDR1/P-gp) is an efflux protein with aberrant activity that has been linked to multidrug resistance in cancer. For the reversal of MDR by RNA interference (RNAi) technology, an U6-RNA gene promoter-driven expression vector encoding anti-MDR1/P-gp short hairpin RNA (shRNA) molecules was constructed (abbreviated pDNA-iMDR1-shRNA). This study explored the feasibility of using Pluronic P123-conjugated polypropylenimine (PPI) dendrimer (P123-PPI) as a carrier for pDNA-iMDR1-shRNA to overcome tumor drug resistance in breast cancer cells. P123-PPI functionalized with anti-CD44 monoclonal antibody (CD44 receptor targeting ligand) (anti-CD44-P123-PPI) can efficiently condense pDNA into nanocomplexes to achieve efficient delivery of pDNA, tumor specificity and long circulation. The *in vitro* studies methodically evaluated the effect of P123-PPI and anti-CD44-P123-PPI on pDNA-iMDR1-shRNA delivery and P-gp downregulation. Our *in vitro* results indicated that the P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes with low cytotoxicity revealed higher transfection efficiency compared with the PPI/pDNA nanocomplexes and Lipofectamine™ 2000 in the presence of serum. The nanocomplexes loaded with pDNA-iMDR1-shRNA against P-gp could reverse MDR accompanied by the suppression of MDR1/P-gp expression at the mRNA and protein levels and improve the internalization and cytotoxicity of Adriamycin (ADR) in the MCF-7/ADR multidrug-resistant cell line. BALB/c nude mice bearing MCF-7/ADR tumor were utilized as a xenograft model to assess antitumor efficacy *in vivo*. The results demonstrated that the administration of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes combined with ADR could inhibit tumor growth more efficiently than ADR alone. The enhanced therapeutic efficacy of ADR may be correlated with increased accumulation of ADR in drug-resistant tumor cells. Consequently, these results suggested that the use of pDNA-iMDR1-shRNA-loaded nanocomplexes may be a promising gene delivery strategy to reverse MDR and improve the effectiveness of chemotherapy.

1. Introduction

Multidrug resistance (MDR) in cancer cells, the phenomenon in which malignant cells frequently become resistant to multiple drugs with unrelated molecular structures, is a major bottleneck of chemotherapy in clinical practice [1–3]. The overexpression of the adenosine triphosphate-binding cassette (ABC) transmembrane transporter protein P-glycoprotein (MDR1/P-gp) is the typical cause of MDR via complicated mechanisms, and this protein is characterized by a typical cross-resistance pattern against anticancer agents [4]. These proteins lead to reductions in the intracellular drug concentration and decreased cytotoxicity due to their ability to pump drugs out of the cells. Therefore, many studies have focused on inhibiting the function of efflux transporters, such as MDR1/P-gp, or their expression to overcome the MDR effect in cancer cells [5–10]. RNA interference (RNAi) technology has been

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widely considered a promising approach for silencing target gene expression efficiently with high specificity at the transcriptional level and reducing the efflux of chemotherapeutic agents [5,11–14]. Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) are ordinarily used by the gene silencing mechanism to inhibit gene expression, which has highlighted their potential use as therapeutic agents in cancer gene therapy. MDR1/P-gp-mediated MDR has been successfully reversed by the application of RNAi technology in different cell models derived from various tumors. shRNA is the precursor of siRNA in the intracellular microenvironment. Moreover, siRNAs can only achieve a transient therapeutic effect, but expression vectors encoding siRNA-like shRNAs have been developed to allow the long-term production of therapeutic RNAs in target cells [15]. For example, Ulrike and colleagues reported that the multidrug resistance phenotype could be reversed completely *in vivo* by the jet-injection of anti-MDR1 shRNA-encoding plasmid DNA. The results demonstrated that shRNA-expressing vectors effectively reverse MDR1/P-gp-mediated MDR *in vivo* and are therefore promising candidates for application to revert tumors with MDR1/P-gp-dependent MDR to a drug-sensitive state [16].

Regardless of the presence of siRNA, shRNA or plasmid DNA (pDNA) can ineffectively bind to cell-surface membranes by themselves, and their subsequent internalization is not possible. The identification of an efficient and safe gene delivery vector remains a major challenge to successful gene therapy. Nanoformulations based on cationic polymeric materials can assemble anionic nucleic acids into nanoparticles via ionic interactions and deliver nucleic acids to mammalian cells, leading to significantly increased transfection efficiency. A variety of cationic polymers have been designed as non-viral vectors and developed for gene delivery [17–23]. Among the different cationic polymers that have been reported, dendrimers have particular advantages [24–27]: (1) Dendrimers are highly branched with complete uniformity and dispersity, a regular structure and a high degree of symmetry; (2) There are a large number of cavities within the dendrimer molecules and a large number of functional groups on the surface of the dendrimer molecules; (3) The nano-size of dendrimers is suitable for cellular uptake and carrying foreign genes into the nucleus; and (4) The significant “proton sponge effect” of the dendrimers can promote the escape of pDNA from the lysosomes and thus avoid its degradation. The typical polycationic dendrimers are polyamidoamine (PAMAM) [28–30] and polypropyleneimine (PPI) [31–33], which have been widely used as carriers for gene delivery. Compared with PAMAM, PPI has not been widely studied. The transfections of G1-5 PPI have been performed in A431 cells, and the results have shown that PPI (G2 and G3) exhibits high transfection efficiency, equivalent to that of the cationic lipid carrier DOTAP, whereas PPI (G4 and G5) alone cannot be used for transfection because of its cytotoxicity [33]. There are two types of amino groups on the surface of G3 PPI, which can condense nucleic acids effectively with slight cytotoxicity and a significant “proton sponge effect”. Thus, G3 PPI was used as the core of the non-viral gene delivery vectors designed in this study.

A key issue of successful non-viral vectors for gene delivery *in vivo* is their stability in the blood circulation. Cationic biomaterials, such as PEI and PAMAM, show high transfection efficiency *in vitro*, but the particle size and dispersion of these polymer vector systems are very sensitive to serum and buffer when applied *in vivo*, resulting in low transfection efficiency, cytotoxicity, poor biodistribution and rapid clearance from the blood, which are mainly attributed to the strong positive charge of cationic polymers. Some hydrophilic polar molecules, such as poly(ethylene oxide) (PEO) [34] and poly(ethylene glycol) (PEG) [35–38], have often been introduced to modify a cationic polymer to inhibit nonspecific protein adsorption by increasing particle stability and circulation time. PEGylated cationic polymers have been extensively studied as gene delivery vehicles. The modified cationic polymer may condense pDNA-forming core–shell nanostructures with a cationic polymer/pDNA core and a non-ionic hydrophilic shell and showed enhanced stability compared with non-PEGylated polyplexes. However, the use of the core–shell systems for gene delivery unavoidably compromised cellular internalization and transfection efficiency. This result is likely due to the hydrophilic shell reducing the interaction between the vector and the cell membrane and thereby resulting in poor cellular internalization [39]. Herein, Nguyen et al. proposed that cationic polymers can be modified with Pluronic rather than PEG [40]. This hypothesis was determined based on the following considerations: As non-ionic surfactants, the hydrophobic chain PO of Pluronic block copolymers has been shown to interact with the plasma membrane, increase the cell membrane fluidity and promote the cellular uptake of various small molecules and biomacromolecules. The EO chain of Pluronic molecules can make polyplexes stable in aqueous medium and reduce their interaction with plasma proteins, thereby protecting the pDNA from nuclease degradation, similarly to the effects obtained with Pluronic micelles [40]. In this manuscript, we describe the Pluronic P123, a triblock copolymer of ethylene oxide (EO) and propylene oxide (PO) EO20–PO70–EO20, not only exhibits important the biological activities mentioned above but also acts as an inhibitor of P-glycoprotein (P-gp), which has been found to recuperate the sensitivity of multidrug-resistant (MDR) tumor cells to doxorubicin, paclitaxel and other anti-cancer agents [41].

To increase tumor-targeting drug delivery, many studies have focused on the molecular markers that are specifically overexpressed in cancerous cells and the tumor microenvironment. The cell surface membrane-bound protein CD44 is most widely overexpressed in various types of cancer cells [42–44] and is particularly interesting for the cancer-selective delivery of chemotherapeutic drugs, peptides and nucleic acid drugs. An antibody that binds to specific cell surface receptors has been well known to allow the insertion of a ligand into carries to improve tumor targeting. Anti-CD44 antibody, which has been identified as a potent tumor-targeting ligand, can recognize CD44 receptors expressed exclusively within solid tumor. P-gp and CD44 are expressed in multidrug-resistant but not in parental, sensitive cell lines [45]. Breast cancer cells overexpressing CD44 receptors tend to be much more elastic than normal cells. The anti-CD44 antibody ligand–receptor system provides a new pathway for breast tumor-targeting diagnosis and therapy and prompts the current interest in the use of CD44 as a very important target for drug development [46–48].

The aim of this study was to re-sensitize doxorubicin-resistant breast cancer cells to the anticancer agent doxorubicin through the selective inhibition of P-gp. This study focused on developing an anti-CD44 antibody-conjugated Pluronic P123-PPI (anti-CD44-P123-PPI)-based nanocarrier to deliver pDNA-iMDR1-shRNA selectively into MCF-7/ADR cells to overcome drug resistance and administered ADR successively to achieve a combinatory effect of chemotherapy and gene therapy. To demonstrate the synergistic effects of gene therapy and chemotherapy, the nanocomplexes loaded with pDNA-iMDR1-shRNA were evaluated based on their suppression of MDR1/P-gp expression at the mRNA and protein levels and the internalization and cytotoxicity of ADR in MCF-7/ADR cells *in vitro*. We also demonstrate the *in vivo* restoration of chemosensitivity to the MDR1/P-gp substrate ADR by delivering nanocomplexes loaded with pDNA-iMDR1-shRNA intravenously into a xenograft tumor model (nude mice). Using a combination of gene therapy and chemotherapy, the therapeutic efficacy is reflected by the knock down of MDR1/P-gp *in vivo*, which