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Research Article

Efficient *In Vitro* TRAIL-Gene Delivery in Drug-Resistant A2780/DDP Ovarian Cancer Cell Line via Magnetofection

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) presents great promise as an anticancer agent for human cancer therapy. In this study, a magnetofection agent (polyMAG-l000) was evaluated for *in vitro* delivery of TRAIL gene towards drug-resistant A2780/DDP ovarian cancer cells. Transfection experiments showed that polyMAG-l000 was able to transfect A2780/DDP cells *in vitro*, leading to a higher level of TRAIL gene expression in the presence of a static magnetic field as compared to other transfection agent, such as Lipofectamine 2000. TRAIL gene expression in the A2780/DDP cells was also confirmed by Western blot analysis. Moreover, the TRAIL gene expression exhibited remarkable decrease in the cell viability, as determined by MTT assay. Importantly, PolyMAG-l000-mediated TRAIL gene transfection in the presence of anticancer drug cisplatin (CDDP) induced much higher percentages of apoptotic A2780/DDP cells, compared to TRAIL gene transfection or CDDP treatment alone. A further study by Western blot analysis indicated that cytochrome *c* release and caspase-9 cleavage pathway were associated with the initiation of the apoptosis in A2780/DDP cells. The results of this study indicate that polyMAG-l000 can be used as an efficient agent for TRAIL gene transfection in ovarian cancer cells.

1. Introduction

Ovarian carcinoma is the fifth most common gynaecological cancer. Due to the lack of effective methods for early diagnosis of ovarian cancer, it is often detected in an advanced stage. As such, the patients usually have to undergo surgery for removal of the tumor and receive chemotherapy using anticancer drugs, such as cisplatin and doxorubicin. In many cases, chemotherapy has to be discontinued due to intrinsic or developed drug resistance of the ovarian cancer cells. To address this critical issue, chemotherapy in combination with biological therapy has been studied in the past decade. The combined approach was proven to be more efficacious than chemotherapy or biological therapy alone. For instance, the combined cisplatin treatment with trastuzumab, a monoclonal antibody capable of interfering with the HER2/neu receptor, may enhance growth suppression of a few types of breast cancer cells [1]. In particular, some recent studies indicated that a combined use of anticancer drugs with TNF-related apoptosis-inducing ligand (TRAIL) protein had a synergistic effect on the apoptotic activity in ovarian cancer cells [2].

TRAIL is a promising anticancer protein which is capable of inducing specific apoptosis in a broad range of cancer cells but not in normal cells [2–5]. However, the clinical use of TRAIL protein is seriously impeded due to its instability *in vivo* and potential resistance of cancer cells to the TRAIL. For enhanced tumor growth inhibition, a high dose of the protein has to be administrated *in vivo*. As an alternative approach, recombinant virus-mediated TRAIL-gene therapy has been investigated recently. This is attributed to the viruses being potent to transfer genes into the somatic cells, yielding high levels of gene expression. Griffith et al. constructed a TRAIL-encoded adenovirus and found that the virus may lead to TRAIL expression in ovarian cancer cell lines (e.g., A2780 and SKOV-3) and to their death mediated

by a caspase-activated apoptosis [3]. These results indicate that TRAIL gene therapy is promising for ovarian cancer therapy. However, there remains serious safety concerns on recombinant viruses as gene vectors for TRAIL-gene transfection.

Nonviral carriers such as cationic polymers for gene transfection have received much attention in the past decades, as they do not present major safety issues compared to other viral vectors. The safety issues of these viral vectors cannot be easily addressed including immunogenicity, toxicity, mutagenicity, and oncogenicity [6–8]. Cationic polymers can condense DNA into nanosized polymer/DNA complexes (polyplexes) via an electrostatic interaction for targeted cells nucleus deliveries. Moreover, the polymers can effectively protect DNA from nuclease degradation in the cells, thereby inducing appreciable transfection efficiency. A variety of cationic polymers, such as poly-l-lysine [9], polyethyleneimine (PEI) [10], polyamidoamine dendrimer [11], and poly(2-dimethylamino)ethyl-methacrylate [12], were studied as gene carriers for nonviral gene delivery. However, these current systems generally induced either low transfection efficiency or high cytotoxicity *in vitro* and *in vivo* [13–15]. It was shown that in the presence of a static magnetic field, polycation-modified magnetic nanoparticles enabled more efficient gene transfection as compared to conventional polymer-based gene delivery systems [16–21]. As a result of externally applied magnetic field, the magnetic nanoparticles/gene complexes can be effectively internalized in the cellular membrane. Therefore, cationic magnetic nanoparticles have been widely employed as new gene delivery systems for magnetofection *in vitro* and *in vivo* [22].

In this study, a commercial magnetofection agent, a cationic magnetic iron oxide (polyMag-1000), was evaluated for TRAIL gene delivery *in vitro* against drug-resistant A2780/DDP ovarian cancer cells, in terms of TRAIL gene transfection efficiency, cell viability, TRAIL gene transfection combined with cisplatin treatment and associated apoptotic mechanism.

2. Materials and Methods

2.1. Magnetic Iron Oxide Nanoparticles and Plasmids. Superparamagnetic iron oxide nanoparticles coated with PEI (polyMAG-1000) were ordered from Chemicell (Germany). Human TRAIL cDNA, kindly donated by Dr. H Yagita (Juntendo University School of Medicine, Japan), was subcloned into the *Eco*RI and BamH I site of the eukaryotic expression vector, pEGFP-N1 plasmid, (Clonetech), yielding the new plasmid pCMV-TRAIL-EGFP. A liposome transfection agent (Lipofectamine 2000) was purchased from Invitrogen (USA).

2.2. Cell Culture. Drug-resistant A2780/DDP cell line was provided by the American Type Culture Collection (ATCC, Manassas, Va, USA). The cells were maintained in DMEM medium supplemented with 10% fetal calf serum (GIBCO Life Technologies, Burlington, ON, Canada).

2.3. Zeta-Potential Measurements and Agarose Gel Retardation. The surface charges of polyMag-1000/plasmid complexes were measured at 25°C using a nanosizer NS90 (Malvern Instruments Ltd., Malvern, UK).

PolyMag-1000/DNA complexes were prepared at varying ratios from 1/8 to 5/1 (w/v). After addition of 2 μL of 6× loading buffer (Fermentas), the mixture was applied onto a 1% agarose gel containing 0.5 μg/mL ethidium bromide. After development of the gel, siRNA was visualized with a UV lamp using a Tanon Gel system (Tanon Gel Image System, Thermo Scientific).

2.4. In Vitro Gene Transfection. A2780/DDP cells (1×10^5) were seeded into 6-well plates and incubated at 37°C in 5% CO_2 for 24 h before transfection. PolyMAG-1000 to DNA ratio is expressed as volume (v) to weight (w) of DNA, and written as v/w. The dose of plasmid DNA used was 3 μg per well. These PolyMAG-1000/DNA complexes were added to 6-wel1 plates after incubation for 30 min at room temperature. A MagnetoFACTOR plate (130 mT) was then placed under each of the 6-well plates for 15 min. After 6 h incubation with serum-free DMEM at 37°C in 5% CO_2, the medium was replaced with fresh DMEM containing 10% FBS and incubated for another 72 h. The polyMAG-1000 without TRAIL gene was served as a negative control. Liposome-mediated TRAIL gene transfection was served as a positive control and performed according to the manufacturer's instructions.

2.5. Flow Cytometry Assay. The Annexin V apoptosis detection kit was used to measure the percentage of apoptotic cells following the manufacturer's protocol (Pharmingen, BD Biosciences, San Diego, USA). Briefly, the TRAIL-gene transfection A2780/DDP cells were washed with PBS and resuspended in 100 μL of binding buffer, and then incubated with 5 μL of FITC-conjugated annexin V for 15 min at room temperature in the dark, followed by the addition of 400 μL of binding buffer. Cells were sorted by a FACScan cytometer using CELLQuest software (version 3.3; BD Biosciences, Mountain View, Calif, USA).

2.6. MTT Assay. Cell metabolic activity, determined by MTT assay, was studied as a function of cell incubation time from 1 day to 4 days. At different time intervals (day 1 to day 4), MTT solution (20 μL, 5 mg/mL) was added to each well and incubated with the cells for 4 h. The medium was then removed and DMSO (150 μL) was added to each well. The absorbance was measured at 570 nm using a plate reader (Thermo scientific). The percentage of relative cell viability was determined relative to control cells (untreated A2780/DDP cells), and taken as 100% cell viability. All cytotoxicity experiments were performed for eight times.

2.7. Western Blot. Proteins were developed by SDS-poly acrylamide gel and then transferred onto cellulose membranes (Millipore, Bedford, Mass, USA). The membranes were first incubated with primary antibodies (anti-TRAIL 1 : 1000) and then incubated with horseradish peroxidase-labeled secondary antibodies. Peroxidase activity was visualized with the enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer's instructions.

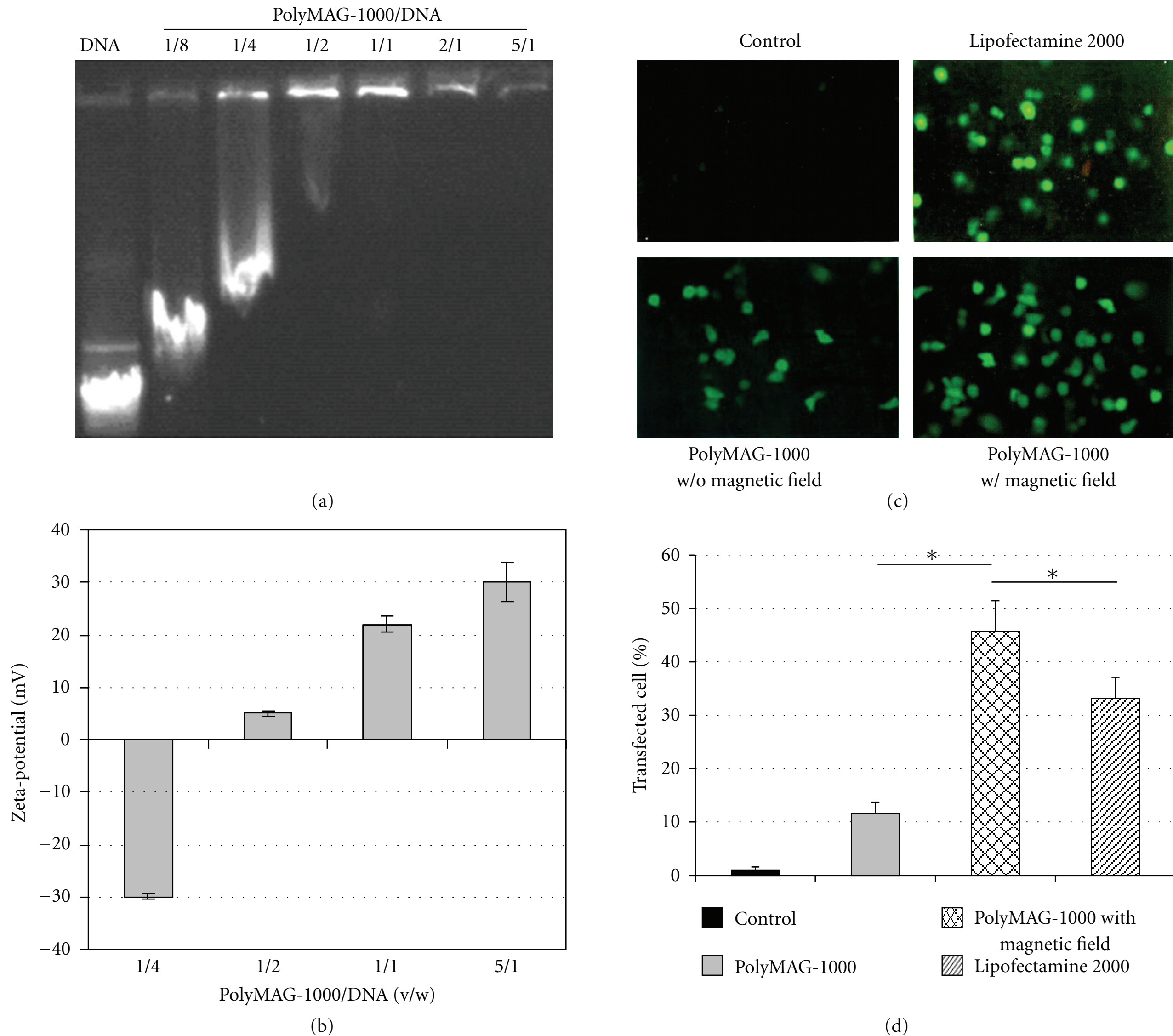


Figure 1: (a) Gel retardation and (b) Zeta-potential analysis on the complexation of polyMAG-1000/plasmid at varying v/w ratios; (c) Effect of a static magnetic field on gene transfection of pCMV-TRAIL-GFP plasmid towards A2780/DDP cells. The A2780/DDP cells were transfected with polyMAG-1000/plasmid, at the v/w ratio of 1/1, in the absence (w/o) or presence (w/) of a static magnetic field. As a control, the A2780/DDP cells were also transfected with lipofectamine 2000. (d) Transfection efficiency was determined by flow cytometry analysis ($^*P < 0.05$). Results are shown as means and SD values of three independent experiments.

Relative band intensity of Western blot was quantified with Adobe Photoshop CS2, 9.0 edition.

2.8. Statistical Analysis. To determine significant differences in the parameters between the sample groups, a paired (for the same sample populations with different treatments) or an unpaired (for different sample population), Student's *t*-test was utilized.

3. Results

3.1. PolyMAG-1000 Mediation of Efficient TRAIL Gene Transfection In Vitro. In this study, PolyMAG-1000, polyethylenimine-modified magnetic nanoparticles, is used as a transfection agent for TRAIL plasmid transfection. It was shown that PolyMAG-1000 is capable of efficiently binding DNA into positively charged complexes [17]. To proof this, gel retardation experiment and zeta-potential analysis were performed. As revealed in Figure 1(a), full DNA retardation was detected at and above the PolyMAG-1000/DNA ratio of 1/1 (v/w). Moreover, the complexes, prepared at the ratio of 1/1, displayed a positive surface charge of about +21 mV (Figure 1(b)). These results thus imply the formation of PolyMAG-1000/DNA complexes.

Magnetofection of polyMAG-1000 against A2780/DDP ovarian cancer cells was studied *in vitro* using pEGFP-N1-TRAIL plasmid, which was encoded with EGFP as a reporter

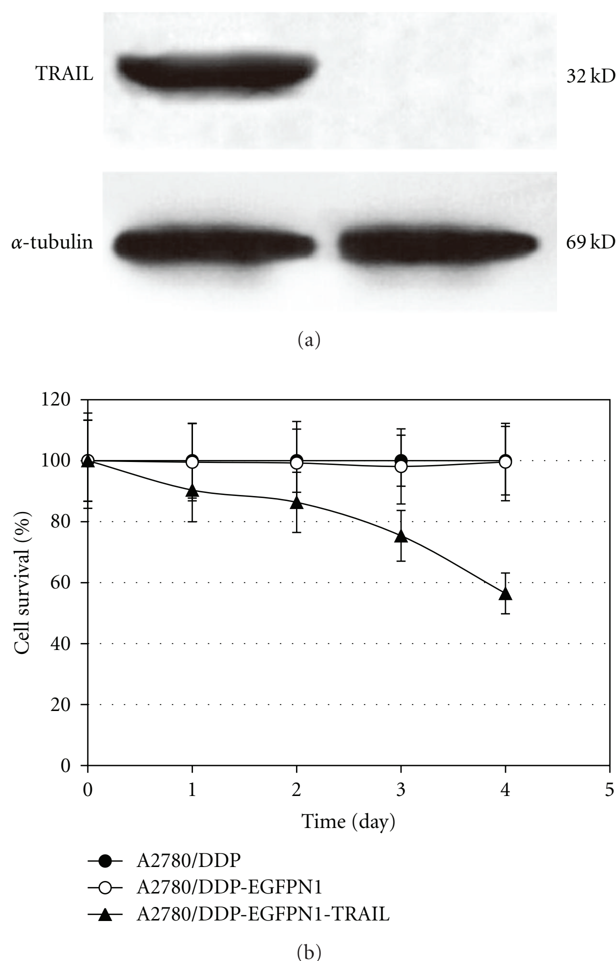


Figure 2: (a) Western blot analysis showing TRAIL gene expression after TRAIL gene transfection via magnetofection in A2780/DDP cells; (b) The effect of TRAIL gene expression on the cell survival rate of A2780/DDP cells ($n = 3$). The cell survival of transfected cells was determined by MTT assay as a functional of transfection time.

gene, at a polyMag-1000/plasmid ratio of 1/1. The gene expression was easily visualized under a fluorescent microscope after 72 h transfection. The percentages of transfected cells were quantified using FACS. As shown in Figure 1(c), polyMAG-l000 is able to efficiently transfect A2780/DDP cells, with transfection efficiency significantly higher in the presence of a static magnetic field than that without the magnetic field (Figure 1(d), 45.67 ± 5.78% versus 11.60 ± 2.12%, $P < 0.05$). As can be seen in this figure, the efficiency is also higher than that with another transfection agent, Lipofectamine 2000, as a positive control (33.14 ± 3.97%, $P < 0.05$).

3.2. TRAIL Expression in A2780 Ovarian Cancer Cells. To further confirm the expression of the TRAIL gene in the A2780/DDP cells after polyMAG-1000 mediated magnetofection, Western blot analysis was performed, and the results are shown in Figure 2(a). A strong immunoreactive protein is observed with a size of 32 kDa. This size is consistent with that of TRAIL in a transmembrane form, indicating successful gene expression of TRAIL in the cells [3, 5, 19].

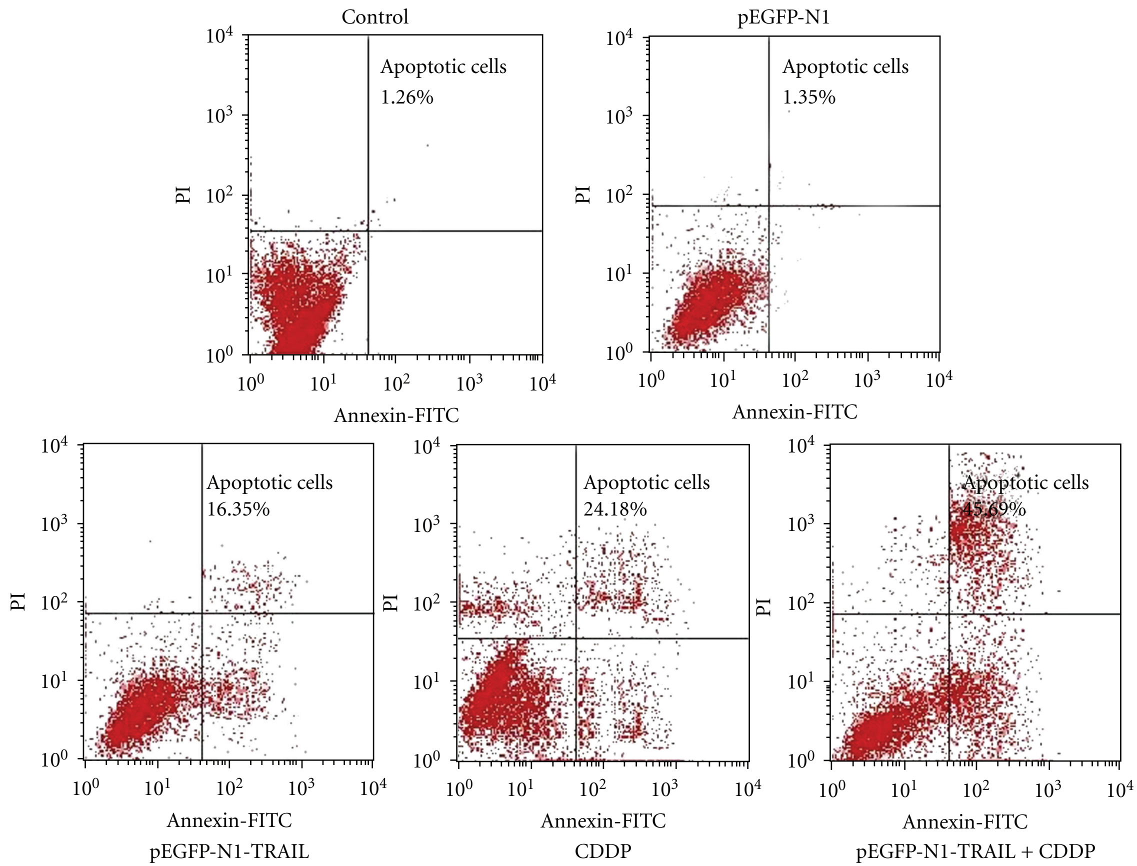
3.3. Human TRAIL Inhibition of A2780/DDP Cells Proliferation In Vitro. Figure 2(b) shows metabolic activities of transfected A2780/DDP cells as a function of transfection time, determined by MTT assay. After 72 h transfection with TRAIL gene, a significant decrease in the metabolic activity is observed for the A2780/DDP transfection by polyMAG-1000/TRAIL compared with A2780/DDP cells by EGFP-N1 plasmid without encoding TRAIL, or untreated A2780/DDP cells as a blank control. These data indicate that TRAIL gene expression in A2780/DDP cell *in vitro* has an inhibitory effect on their survival activity.

3.4. TRAIL Enhances the Sensitivity of A2780 Cells to CDDP. To evaluate the effect of anticancer drug CDDP on apoptosis in TRAIL-transfected A2780/DDP cells, polyMAG-1000-induced TRAIL transfection was carried out in the presence of CDDP (20 μM). The percentage of apoptotic cells was quantified using an annexin V-FITC staining assay, which can report the loss of phosphatidylserine asymmetry of the plasma membrane at an early stage of apoptosis. It is found that TRAIL gene transfection in the presence of CDDP exhibits statistically higher percentages of apoptotic cells, as compared to TRAIL gene transfection or CDDP treatment alone (Figure 3(b), 45.69 ± 9.21% versus 16.35 ± 3.24% or 24.18 ± 5.87%, resp., $P < 0.05$,).

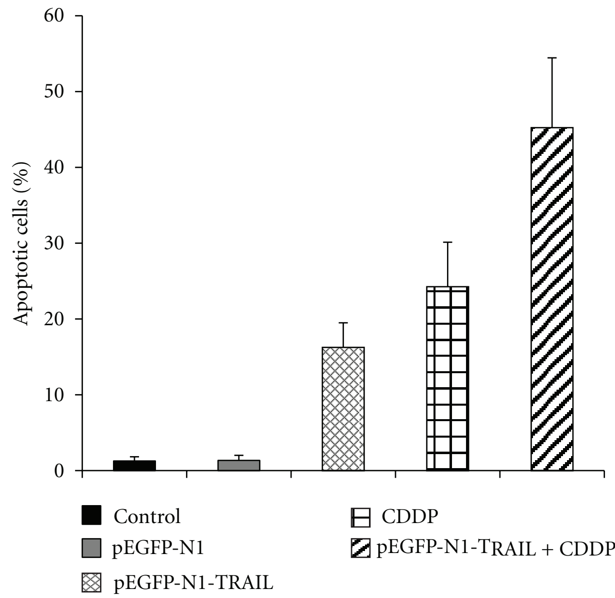
3.5. Cytochrome c Release and Cleaved Caspase-9 Activation Involving TRAIL Transfected A2780/DDP Cells. Cytochrome *c* release and caspase-9 activation have been described as the terminal operation in cell apoptosis [23–25]. To understand cytochrome *c* release and cleaved caspase-9 activation involved in the apoptotic A2780/DDP cells, subcellular cytochrome *c* release and the activation of caspase-9 were studied by Western blot analysis (Figure 4(a)). The activation of caspase-9 was confirmed by detecting the generation of 37 kDa proteolytic fragment of caspase-9. It is shown that as compared to untreated A2780 cells, cytochrome *c* level increases for both TRAIL transfected and CDDP-treated A2780/DDP cells. Moreover, the combined TRAIL transfection and CDDP treatment induces upregulation of both cytochrome *c* and caspase-9 activation in A2780/DDP cells (Figure 4(b)).

4. Discussion

The key challenge for efficient nonviral gene delivery is to design potent carriers capable of overcoming multiple intracellular barriers, such as plasmid internalization, endosomes, cytoplasm trafficking, nuclear translocation, as well as the retention of plasmid in the nucleus [26–32]. PolyMAG-l000 has shown great promise for TRAIL plasmid transfection since it is capable of binding plasmid into positively charged complexes (Figures 1(a) and 1(b)), and also promoting efficient cellular internalization of polyplexes in the presence of a static magnetic field [17, 18]. This may explain the



(a)



(b)

Figure 3: Treatment with CDDP induces apoptosis in TRAIL transfected drug-resistant A2780/DDP cells. (a) Histograph analysis of typical flow cytometry data to determine the percentages of apoptotic A2780/DDP cells after CDDP treatment, TRAIL gene transfection, or the combined, using Annexin V-FITC/PI staining assay. (b) Quantification data of the percentages of apoptotic A2780/DDP cells. The means and errors results were calculated from three independent experiments.

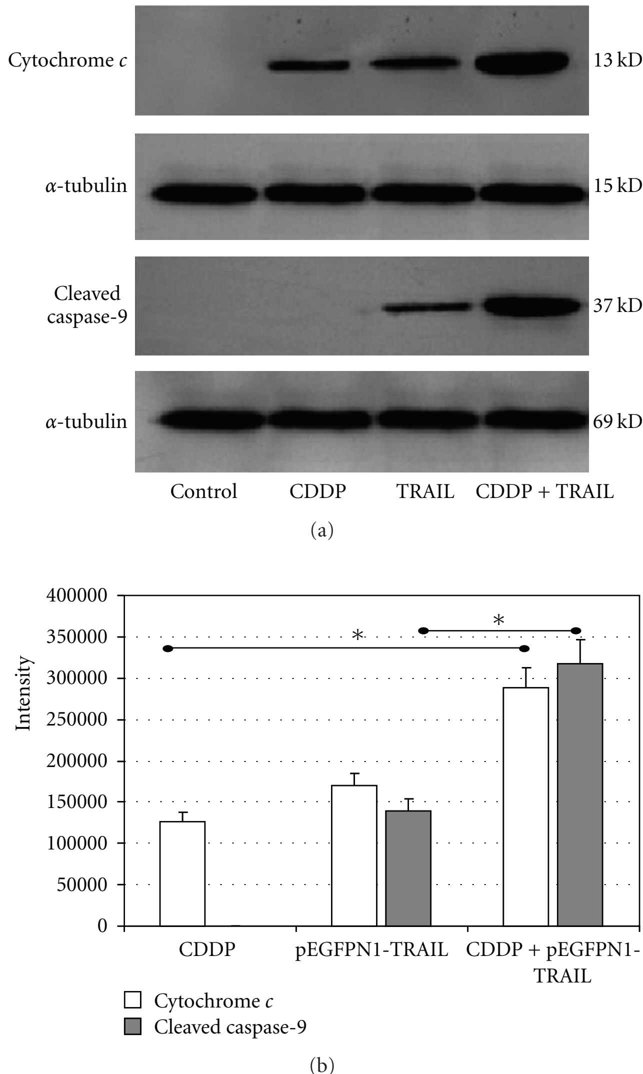


Figure 4: Combination of CDDP treatment and TRAIL transfection enhances cytochrome *c* release and cleaved caspase 9 in A2780/DDP cells. (a) Western blot analysis showing the cytochrome *c* expression and cleaved caspase 9 in A2780/DDP after CDDP treatment (20 μM), TRAIL gene transfection, or the combined TRAIL transfection and CDDP treatment. An anti-α-tubulin was used as a reference control. (b) Quantified absolute intensity of the bands in the Western blot analysis ($P < 0.05$).

fact that more efficient TRAIL-plasmid expression mediated by polyMAG-1000 magnetofection was observed in the presence of a static magnetic field and that the transfection efficiency is higher than that of Lipfectamine 2000, as a positive control (Figures 1(c) and 1(d)). PolyMAG-l000 has been previously used for plasmid magnetofection in various cell lines such as MCF-7 and stem cells [33]. However, to our knowledge, this is the first paper on TRAIL plasmid magnetofection in ovarian cancer cells.

TRAIL protein can lead to specific apoptosis in a broad range of cancer cells, such as melanomas, gliomas, and prostatic carcinomas rather than the normal cells [3]. Thus, TRAIL is expected as a potential therapeutic agent for ovarian cancer treatment. In this study, the TRAIL gene is found to induce apoptosis in drug-resistant A2780/DDP cells (Figure 3), which thus contributes to the decrease in the cell viability of the cells after 3-day transfection (Figure 2). Moreover, the TRAIL expression enhanced the sensitivity of the A2780/DDP cells to the anticancer drug CDDP. A higher percentage of apoptotic A2780/DDP cell was found when the cells were cotreated by both TRAIL transfection and cisplatin. The results thus imply that TRAIL gene therapy in combination with CDDP treatment is an efficient approach for the treatment of drug-resistant ovarian cancer. Further study will be focused on the combined use of the TRAIL magnetofection and CDDP treatment in the xenograft A2780/DDP tumor in a nude mouse model.

Two viable approaches of caspase activation have been identified [25, 34]. The activation of initiator caspase-8 or caspase-10 is triggered by the ligation of death receptors, such as Fas, TNFR1, or Death Receptor 3. The second one pathway is essentially controlled by mitochondria. In most cases, the apoptotic cascade is initiated by loss of integrity of the outer mitochondrial membrane accompanied by release of cytochrome *c*. Cytochrome c, released from mitochondria induces a conformational change in the apoptotic protease activating factor (Apaf-I). This mechanism results in the cleavage and activation of caspase-9 and the subsequent activation of a caspase cascade is responsible for the cell death [34]. In this study, enhanced cytochrome *c* and cleaved caspase-9 product are achieved in the A2780/DDP cells by combining the TRAIL transfection and CDDP.

5. Conclusions

We have demonstrated that PolyMag1000 is potent to transfection of drug-resistant A2780/DDP cells *in vitro*. TRAIL gene expression induces growth inhibition of A2780/DDP cell via activating cell apoptosis. PolyMag1000-mediated TRAIL transfection in combination with cisplatin treatment may lead to augmented apoptosis in A2780/DDP cells *in vitro*. These results indicate that PolyMag1000 is an efficient nonviral TRAIL-gene carrier for ovarian cancer therapy.

Acknowledgments

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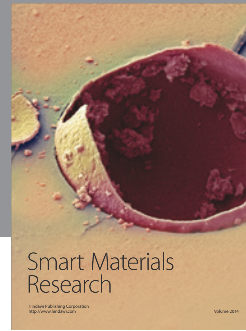
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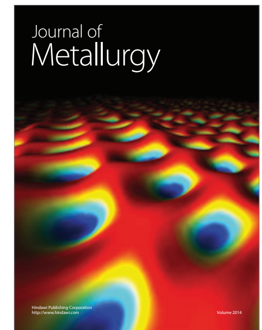
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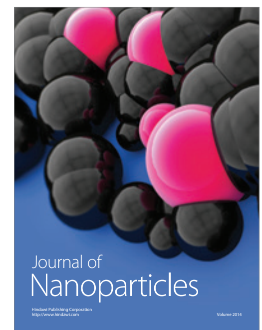
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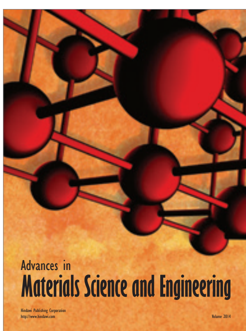
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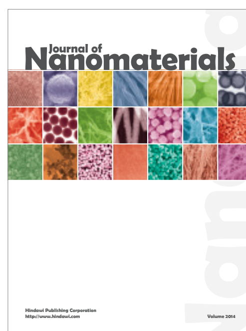
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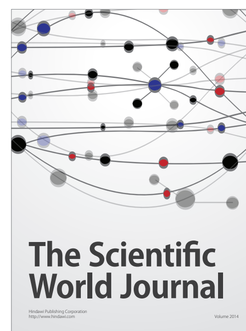
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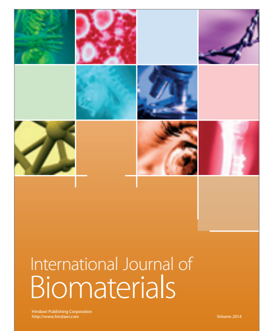
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