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Original Article

Gold nanoparticles conjugated to the secretable trimeric TRAIL gene promote apoptosis in heat-shocked hepatoma cells

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Abstract: Radiofrequency ablation is widely accepted as a curative therapeutic method for liver cancer, but the high rate of recurrence severely affects its efficacy. Gold nanoparticles are designed to deliver drugs and genes to enhance hyperthermia-based treatments. To determine whether gold nanoparticles conjugated to the secretable trimeric tumour necrosis factor-related apoptosis-inducing ligand (stTRAIL) gene can promote apoptosis in heat-shocked hepatoma cells, we combined polyethyleneimine (PEI)-capped gold nanoparticles (AuNPs) with recombinant pIRES2-EGFP-stTRAIL plasmid DNA in a 5:1 weight ratio and used these complexes in combination with heat shock to treat hepatoma cells. The effects on proliferation and apoptosis were examined by CCK-8 assay and flow cytometry. The PEI-capped AuNPs were stable and had good biocompatibility. Combining stTRAIL-AuNP complexes with heat shock to treat liver cancer cells significantly enhanced the inhibition of cell proliferation and promoted apoptosis. The mRNA and protein levels of stTRAIL, Caspase-8 and Caspase-3 were determined by quantitative real-time PCR and western blot to elucidate the mechanism of the apoptosis increase in the stTRAIL-AuNP complex group. Both mRNA and protein levels of stTRAIL, Caspase-8 and Caspase-3 were significantly higher in the experiment group than those in the control group. Our results indicate that stTRAIL-AuNP complexes may have a potential role in improving the efficacy of RFA for the treatment of liver cancer.

Keywords: Liver cancer, TRAIL, gold nanoparticles, heat shock, apoptosis

Introduction

Liver cancer is the sixth most prevalent cancer and the second most common cause of cancer-related death worldwide, severely affecting public health [1]. Hepatic resection, liver transplantation and local ablation are currently the main curative therapies for liver cancer. As the majority of liver cancer patients are diagnosed at an advanced stage, only 10-20% of patients with liver cancer are eligible for surgical resection [2]. Local ablation, such as radiofrequency ablation (RFA), is widely accepted as a safe and effective treatment option for unresectable and small liver cancers (≤3 cm) [3]. The efficacy of RFA for tumours smaller than 3 cm has been reported to be comparable to that of surgical resection [4]. Additionally, RFA has various advantages such as minimal invasiveness, repeatability, low cost and short hospitalization time [5]. However, complete ablation is difficult to achieve in some tumours due to irregular morphology and tumour positions adjacent to large blood vessels, the intestine or gallbladder. Local recurrence often occurs at the transition zone where the temperature (60-42°C) is too low to induce cell apoptosis or death [6]. Until recently, effective methods to manage residual tumours and local recurrence have been lacking.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane cytokine molecule of the TNF family. TRAIL induces apoptosis in a wide variety of cancer cells, including MDA-MB-231, A549 and U-87G cells [7-9]. Soluble TRAIL has strong selectivity for suppressing tumour growth without damaging normal cells and tissues *in vitro* and *in vivo* [10]. However, soluble TRAIL cannot form the trimer

quaternary structure required for apoptotic activity [11]. The secretable trimetric TRAIL gene (stTRAIL) induces higher apoptotic activity than TRAIL, suggesting that stTRAIL may have potential use as a therapeutic gene for treating liver cancer.

Gold nanoparticles (AuNPs) have attracted increasing interest in biomedical applications because of their unique properties, such as simple synthesis, low toxicity and good biocompatibility [12]. AuNPs are designed to deliver drugs and genes and to enhance hyperthermia-based treatments [13, 14].

In the present study, cultured cells were subjected to heat-shock treatment (47°C, 10 min) to simulate the transition zone of RFA [5, 15]. The effects of stTRAIL-AuNP complexes (recombinant plasmid DNA pIRES2-EGFP-stTRAIL bound to AuNPs) on the apoptosis of heat shock-treated hepatoma cells were evaluated. We further studied the underlying mechanisms and demonstrated the potential use of stTRAIL-AuNP complexes in improving the efficacy of RFA for liver cancer.

Material and methods

Synthesis and characterization of PEI-capped AuNPs

Branched polyethyleneimine (PEI) (molecular weight, 25 kDa) and $HAuCl_4 \cdot 3H_2O$ were purchased from Sigma-Aldrich (Darmstadt, Germany).

To prepare PEI-capped AuNPs, 25 ml of 1.4 mM aqueous $HAuCl_4$ was mixed with 0.9 ml of 1% (w/w) PEI and stirred vigorously at room temperature for 24 h. UV-Vis spectra of the solution were obtained using a Thermo Scientific Varioskan® Flash UV-Vis near-infrared spectrophotometer (Thermo Fisher Scientific Inc., USA). Transmission electron microscopy (TEM) measurements were performed on a JEOL-1400 plus transmission electron microscope with an accelerating voltage of 80 kV (JEOL Ltd., Tokyo, Japan). The particle size, size distribution and zeta potential of the PEI-capped AuNPs in aqueous solution were measured by dynamic light scattering (DLS) on a Malvern Zetasizer Nano S90 instrument (Malvern Instruments Ltd., Malvern, United Kingdom).

Construction and identification of the recombinant pIRES2-EGFP-stTRAIL eukaryotic expression vector

The recombinant plasmid DNA pIRES2-EGFP-stTRAIL was purchased from BGI Gene Company (BGI Gene Company, Beijing, China), amplified in *E. coli* DH5α competent cells, and isolated using an Omega E.Z.N.A. Endo-free Plasmid Mini Kit I (Omega Bio-tek, Inc., Georgia, USA). The correct pIRES2-EGFP-stTRAIL plasmid sequence was verified by restriction enzyme mapping and DNA sequencing.

Plasmid DNA binding stability of PEI-capped AuNPs

PEI-capped AuNPs were mixed with pIRES2-EGFP-stTRAIL plasmid DNA (1 μl, 500 ng/ml) at various weight ratios of AuNPs to DNA. After 10 min of incubation, the agarose gel retardation assay was performed as follows: 6 μl of well-mixed DNA/AuNP complex solution was mixed with 1 μl of 6× loading buffer (TaKaRa Biotechnology, Dalian, Liaoning Province, China); then, 7 μl of the mixture was loaded onto a 1% agarose gel containing 0.1 μl/ml GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., California, USA). Electrophoresis was performed at a voltage of 120 V for 35 min in 1× TAE running buffer. Finally, the results were recorded at 254 nm using a Bio-Rad Gel Documentation Systems instrument (Bio-Rad Laboratories, Inc., California, USA).

Cell culture and cytotoxicity evaluation

The established human HCC cell line SMMC-7721 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were seeded in 96-well plates at 1×10^4 cells per well. After incubated for 24 h, different volumes of PEI-capped AuNPs or equal volumes of PBS in DMEM with FBS were used to replace the culture medium, and the cells were further incubated for 24 h. Next, 10 μl of CCK-8 (ATGene Biotech co., Ltd, Chongqing, China) was added to each well and incubated at 37°C for 2 h. The absorbance value (OD) of each well at 562 nm was measured using a microplate reader (Thermo Scientific, USA). The results were expressed as the mean percent-

age of cell viability relative to untreated cells. The experiments were repeated three times.

Cell proliferation assay

To analyse the effects of stTRAIL-AuNP complexes on the proliferation of heat shock-treated SMMC-7721 cells, CCK-8 assays were performed. Cells were seeded at a density of 5×10^3/well in 96-well plates. After 24 h of incubation, the culture medium was replaced with stTRAIL-AuNP complexes or equal volumes of PBS in DMEM with FBS, and the cells were further incubated for 24 h. After 24 h of incubation, the cells were exposed to heat stress. Heat treatments were performed by sealing the tops of the culture plates with parafilm and submerging the plates in a water bath set to 47°C for 10 min, followed by further incubation. Cell viability was measured using CCK-8 assays as described above at 0 h, 24 h, 48 h, and 72 h after the heat shock treatment.

Apoptosis assay

To examine the effects of stTRAIL-AuNP complexes on the apoptosis of heat-shocked SMMC-7721 cells, apoptosis was assessed using an Annexin V-FITC/PI Apoptosis Detection Kit (Kaiji, Nanjing, China). SMMC-7721 cells were seeded in 6-well plates at a concentration of 5×10^5/well and incubated overnight. Next, the cells were treated with or without stTRAIL-AuNP complexes and incubated for 24 h. Then, the plates were sealed and submerged in a water bath at 47°C for 10 min, followed by incubation for another 48 h. The cells were harvested, washed twice, and stained with Annexin V/FITC and PI according to the manufacturer's instructions. Then, flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Real-time PCR assay for stTRAIL, Caspase-8 and Caspase-3

SMMC-7721 cells were seeded into a 25-cm^2 rectangular canted neck cell culture flask (Corning Incorporated, USA) at 1×10^6/flask and incubated overnight. Next, the cells were treated with or without stTRAIL-AuNP complexes and incubated for 24 h. The plates were then sealed and submerged in a water bath at 47°C for 10 min, followed by incubation of the cells for another 48 h.

Total mRNA was extracted using RNAiso Plus according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Real-time experiments were conducted in triplicate on a CFCX96™ System (Bio-Rad, USA) using the SYBR® Premix Ex Taq™ II Kit with specific primers. The sequences of the primers used to determine the expression of the target gene were as follows: stTRAIL 5'-GACCAGAGGAAGAAGCAACACA-3' (forward) and 5'-GCTCAGGAATGAATGCCCACT-3' (reverse); Caspase-8 5'-CTGGACTACATTCCGCAAAGG-3' (forward) and 5'-GATTGCTTTCCTCCAACATTCTC-3' (reverse); Caspase-3 5'-TGGCATTGAGACAGACAGTGGT-3' (forward) and 5'-TGGCACAAAGCGACTGGATG-3' (reverse); and β-actin 5'-CCACGAAACTACCTTCAACTCC-3' (forward) and 5'-GTGATCTCCTTCTGCATCCTGT-3' (reverse). The PCR parameters consisted of 30 s at 95°C; 40 cycles of denaturation for 5 s at 95°C and annealing for 30 s at 60°C; and primer extension for 10 s at 95°C. The comparative Ct method was used to quantitate the expression of stTRAIL, Caspase-8 and Caspase-3 using β-actin as the normalization control.

Western blot assay for stTRAIL, Caspase-8 and Caspase-3

Transfected cells obtained under conditions similar to those described above were lysed and centrifuged. The supernatants were collected, and the protein concentration was measured using a BCA protein assay kit. Supernatants were loaded on a 10% SDS-PAGE gel and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 2 h and then incubated with their respective primary antibodies overnight, followed by incubation with HRP-conjugated secondary antibodies for 2 h at room temperature. The bands were visualized using an ECL detection kit (Millipore, USA) and analysed using Image-Pro Plus 6.0 analysis software (Media Cybernetics, Bethesda, MD, USA). The following antibodies were used for western blotting: anti-stTRAIL (1:500, sc-7877, Santa Cruz Biotech, USA), anti-Caspase-8 (1:500, sc-5263, Santa Cruz Biotech, USA), anti-Caspase-3 (1:500, sc-7148, Santa Cruz Biotech, USA), and anti-β-actin (1:1000, TA-09, Zhongshajinqiao, China). β-actin was used as internal control.

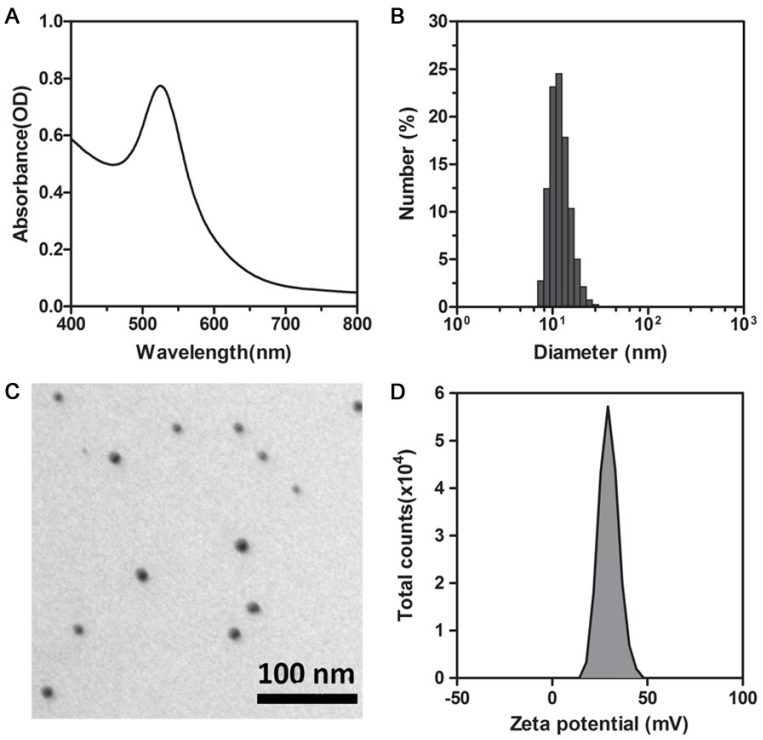

Figure 1. Characterization of PEI-capped AuNPs: A. UV-Vis absorption spectrum of AuNPs; B. Size distribution of AuNPs; C. TEM images of AuNPs; D. Zeta potential of AuNPs.

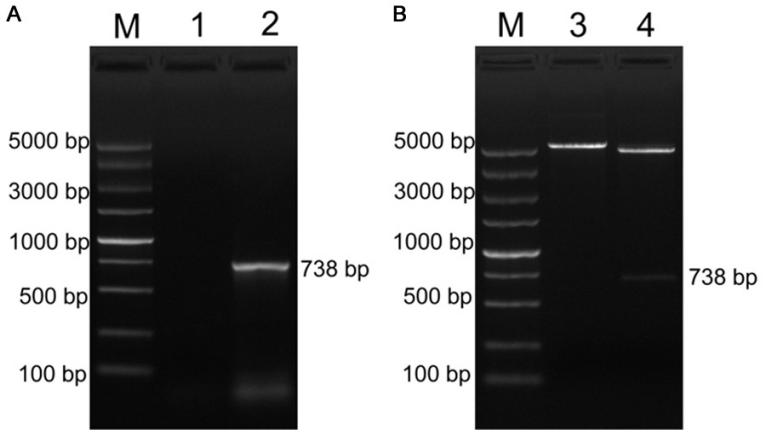

Figure 2. Identification of the recombinant pIRES2-EGFP-stTRAIL plasmid DNA. A. Electrophoresis of the RT-PCR product of the full-length target gene: M: DNA Marker DL5000; 1: negative control; 2: stTRAIL. B. *NheI* and *BamHI* digestion and electrophoresis of the pIRES2-EGFP-stTRAIL eukaryotic expression vector: M: DNA Marker DL5000; 1: pIRES2-EGFP plasmid vector digested by *NheI* and *BamHI*; 2: pIRES2-EGFP-stTRAIL plasmid digested by *NheI* and *BamHI*.

Statistical analysis

The data are presented as the mean ± SD of three independent experiments. Statistical comparisons between two groups were performed using Student's t test. Statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was set as the level of statistical significance.

Results

Characterization of PEI-capped AuNPs

PEI-capped AuNPs were synthesized by directly mixing linear PEI and $HAuCl_4$ aqueous solution at room temperature for 24 h as described previously [16, 17]. Linear PEI with a molecular weight of 25 kDa served as the reductant and stabilizer. After vigorous stirring overnight, the colour of the solution mixture changed from pale brown to wine red, indicating the formation of AuNPs. The peak of the plasmon resonance band of the AuNPs was located at approximately 520 nm, consistent with previous studies [16, 18] and confirming the formation of AuNPs (**Figure 1A**). The hydrodynamic diameter of the PEI-capped AuNPs was measured by DLS, and the average diameter was 12.3±3.3 nm, as shown in **Figure 1B**. The morphology of the PEI-capped AuNPs was characterized by TEM, as shown in **Figure 1C**. The morphology of the nanoparticles was uniform and spherical, and the diameter of the observed nanoparticles was consistent with the DLS measurement. As expected, the surfaces of the AuNPs were positively charged, and the zeta potential was + (29.7±5.1) mV (**Figure 1D**), which suggested that PEI was successfully attached to the AuNP surfaces. The cationic charge density keeps the NPs apart and ensures that biological macromolecules with negative charges, such as

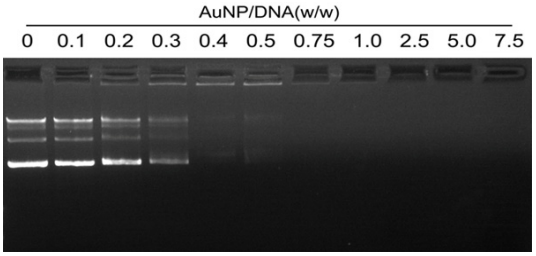

Figure 3. Agarose gel electrophoresis retardation assay of recombinant plasmid DNA/AuNPs. The numbers denote the AuNP to DNA weight ratio.

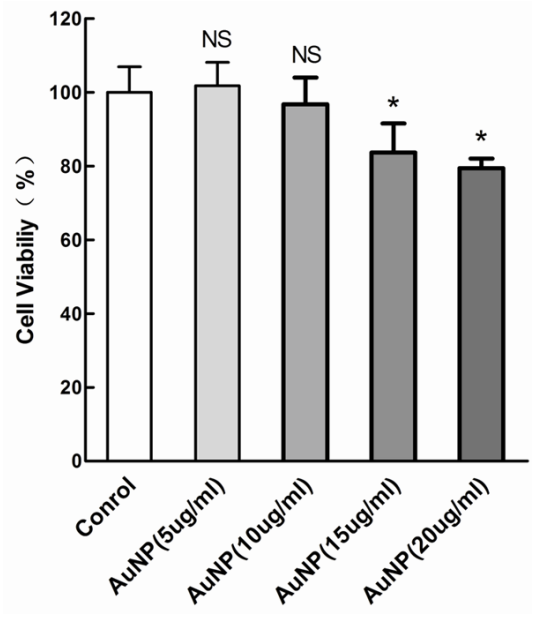

Figure 4. Cytotoxicity of PEI-capped AuNPs to heaptoma cells. (NS, no significance, P>0.05, *, P<0.05, vs control group, Student's t test).

DNA, RNA and proteins, will be attracted to the surfaces of the AuNPs. The mixture was stored at 4°C, and the colour showed no change after six months, indicating that the mixture was stable.

Identification of RT-PCR products and the recombinant pIRES2-EGFP-stTRAIL eukaryotic expression vector

The RT-PCR products were loaded on 1% agarose gels, and the band for full-length stTRAIL was observed at 738 bp (**Figure 2A**). After the stTRAIL cDNA fragment was inserted into the pIRES2-EGFP plasmid (5308 bp), the fragment was confirmed by *BamHI* and *NheI* digestion

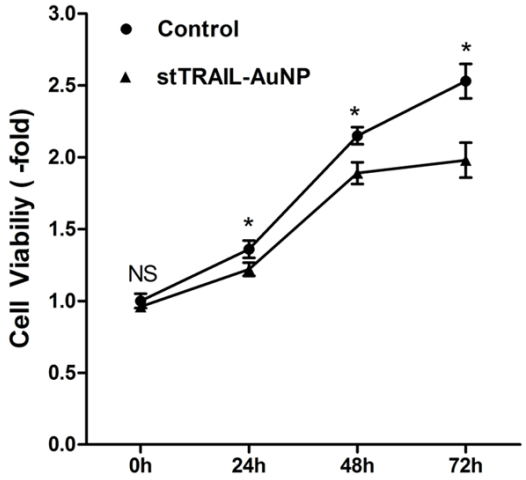

Figure 5. Viability of SMMC-7721 cells after heat-shock treatment. The SMMC-7721 cells were cultured after heat treatment at 47°C. The cell viability of SMMC-7721 cells with or without stTRAIL-AuNP complexes were measured at 0 h, 24 h, 48 h, and 72 h using the CCK-8 assay. (NS, no significance; *, P<0.05, stTRAIL-AuNP group vs control group, Student's t test).

and electrophoresis (**Figure 2B**). Additionally, the cDNA was confirmed by DNA sequencing.

These experiments definitively confirmed that the recombinant pIRES2-EGFP-stTRAIL eukaryotic expression vector was constructed successfully.

Characterization of the PEI-AuNPs/DNA complex

PEI-AuNPs can form nanocomplexes with plasmid DNA through electrostatic interactions between the negatively charged phosphate backbone and the positively charged PEI on the surface. When the weight ratio of AuNPs to DNA was higher than 0.5, the electrophoretic mobility of the DNA was completely retarded (**Figure 3**), demonstrating successful DNA binding to the AuNPs via electrostatic interactions.

Cytotoxicity evaluation

Low cytotoxicity is a prerequisite for vectors used for gene delivery. The cytotoxicity level induced by the various drug combinations was measured using the CCK-8 assay. The cytotoxicity was enhanced as the concentration of PEI-capped AuNPs increased (data not shown in this paper). When the concentration of PEI-capped AuNPs was less than 10 μg/ml, the cell

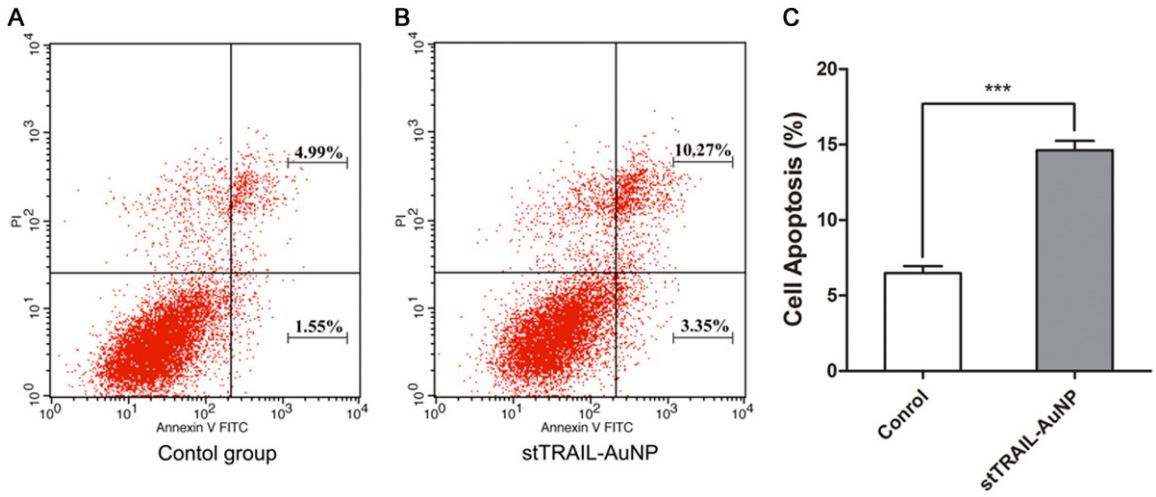

Figure 6. Flow cytometry assay of apoptosis of heat shock-treated SMMC-7721 cells: A. Control group. B. stTRAIL-AuNP complex group. C. Bar chart of the cell apoptosis of the two groups. (***, P<0.001, Student's t test).

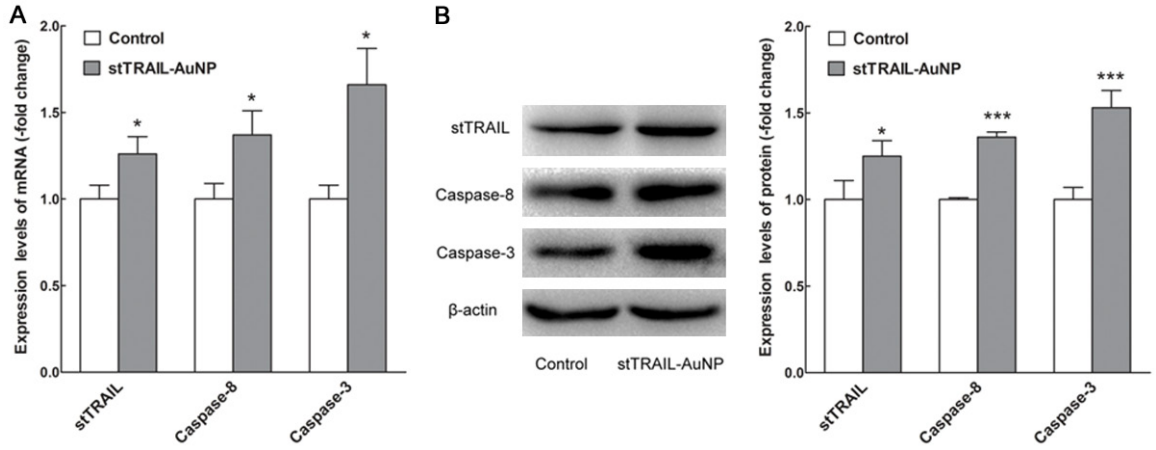

Figure 7. The relative mRNA and protein expression levels of stTRAIL, Caspase-8 and Caspase-3 were measured by quantitative real-time PCR and western blotting. A. Relative mRNA expression levels. B. Relative protein expression levels. (*, P<0.05, ***, P<0.001, Student's t test).

viability did not significantly differ between the PEI-capped AuNPs group and the blank control group (P>0.05) (**Figure 4**). The results of the CCK-8 assay were in good agreement with the cell morphology observed by microscopy. Thus, we chose an AuNP concentration of 10 μg/ml and an AuNP/DNA (w/w) ratio of 5.0 for subsequent experiments.

stTRAIL-AuNP complexes inhibit the proliferation of heat-shocked SMMC-7721 cells

To monitor the potential effect of the stTRAIL-AuNP complexes on the proliferation of tumour cells, we studied the cell viability at 0 h, 24 h, 48 h, and 72 h of SMMC-7721 cells exposed to 47°C for 10 min. We observed a significant decrease in the cell viability of the stTRAIL-AuNP group compared with the control group without stTRAIL-AuNP at 72 h due to cell apoptosis and death (P<0.05) (**Figure 5**).

stTRAIL-AuNP complexes promote the apoptosis of heat-shocked SMMC-7721 cells

To investigate the effects of the stTRAIL-AuNP complexes on apoptosis of heat-shocked SMMC-7721 cells, flow cytometry was performed. Apoptosis of heat-shocked SMMC-7721 cells increased significantly in the group treated with stTRAIL-AuNP complexes compared to the control group (P<0.001), as shown in **Figure 6**.

mRNA and protein expression levels of stTRAIL, Caspase-8 and Caspase-3 in heat-shocked SMMC-7721 cells

Forty-eight hours after the heat treatment, the expression levels of stTRAIL, Caspase-8 and Caspase-3 were analysed by quantitative real-time RT-PCR and western blotting. The expression of stTRAIL, Caspase-8 and Caspase-3 was higher in the stTRAIL-AuNP group than that in the control group ($P<0.05$), as shown in **Figure 7A** and **7B**. This finding indicated that the stTRAIL-AuNP complexes may promote the apoptosis of heat-shocked SMMC-7721 cells though the TRAIL-induced apoptosis pathway. This effect may have a potential role in improving the efficacy of RFA against liver cancer.

Discussion

RFA is widely accepted as a curative therapeutic method for liver cancer in addition to hepatic resection and liver transplantation [3]. However, complete ablation is difficult due to the limitations of the technique. Insufficient ablation leads to local recurrence [19]. Tumour size is an important factor. Insufficient ablation seriously affects the efficacy of RFA for liver cancer. Local recurrence is an urgent problem that requires a solution for the treatment of liver cancer by RFA.

In the present study, we focused on combining stTRAIL-AuNP complexes with heat shock to enhance the inhibition of cell proliferation and promote apoptosis in heat-treated liver cancer cells in the RFA transition zone. This process is an effective method to increase the coagulation volume of RFA by altering tissue properties such as thermal and electric conductivities. Ahmed [20] reported that pretreatment with an intratumoural injection of small volumes of highly concentrated saline solution increased RF heating and coagulation in a canine model. These results suggested that AuNPs may have a potential role in increasing direct thermal injuries when combined with RFA treatment. However, this possibility must be confirmed by additional experimental studies.

Here, we simulated the microenvironment of the transition zone in vitro by submerging the cells in a water bath at 47°C for 10 min, as described previously [15], and the impact of the stTRAIL-AuNP complexes on the apoptosis of the heat shock-treated hepatoma cells was determined. First, we constructed the stTRAIL-AuNP complexes as described above. Then, we confirmed that the PEI-capped AuNPs exhibited good biocompatibility. The cytotoxicity of the PEI-capped AuNPs was not significant ($P>0.05$) when the stTRAIL-AuNP complexes were produced using a 5:1 weight ratio, similar to the results of other studies [16, 18, 21]. The stTRAIL-AuNP complexes significantly inhibited the proliferation of heat-shocked hepatoma cells, especially at 72 h after heat treatment ($P<0.01$). These results indicated that recombinant plasmid DNA encoding the stTRAIL gene can be transfected into hepatoma cells through PEI-capped AuNPs and promote apoptosis in tumour cells. One possible explanation for these observations is that the AuNPs increased the thermal and electric conductivities of the tissue. An alternative explanation is that overexpression of stTRAIL induced apoptosis.

To elucidate the underlying mechanisms, we further investigated the mRNA and protein expression levels of the TRAIL-induced apoptosis pathway. The mRNA and protein expression levels of stTRAIL, Caspase-8 and Caspase-3 were significantly increased in the stTRAIL-AuNP group.

Although the mechanisms underlying local recurrence after RFA remain unclear, our results provide a new method for improving the efficacy of thermal ablation by increasing apoptosis in the RFA transition zone. Our study also has some limitations. We studied only the effects of stTRAIL-AuNP complexes on the apoptosis of hepatoma cells in vitro, but the safety and efficacy of stTRAIL-AuNP complexes for the thermal ablation treatment of liver cancer in vivo have not been tested. Further studies are needed to confirm our findings.

The present study demonstrated that stTRAIL-AuNP complexes prepared by mixing PEI-capped AuNPs with recombinant plasmid DNA pIRES2-EGFP-stTRAIL promoted the apoptosis of heat shock-treated SMMC-7721 cells by upregulating the TRAIL-induced apoptosis signalling pathway. Our results indicate that stTRAIL-AuNP complexes may have a potential role in improving the efficacy of RFA for the treatment of liver cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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