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Original Article

Evaluation of the Cell Death Induction of Gold Nanoparticles Conjugated Antibodies Produced Against a Small Epitope of DR5 Protein in MCF7 Cells

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Abstract

Introduction: Nowadays, versatile and useful features of nanoparticles, especially gold nanoparticles in medicine and healthcare have brought them immense popularity. The ability to transfer towards the special cells, distinguish the different cells and their electrical resonance feature make them as a proper candidate for treatment of cancer. Antibodies which are generated against death receptor, DR5, are powerful tools in the programmed death of cancer cells during induction process. Its association with nanoparticles could efficiently deliver such biological apoptosis inducing drug to the cancer cells

Materials and Methods: In this study, at the first step, gold nanoparticles were produced by chemical methods in the presence of aspartic acid (amino acid). Then, nano-sized ones were selected and subsequently conjugated by mouse antibodies which were produced against a small 21 amino acid peptide from extracellular domain of death receptor, DR5.

Results: The conjugated antibodies by gold nanoparticles could efficiently kill the MCF7 breast cancer cells through inducing cell death. The combination of antibodies which were generated against a small fragment of the death receptor,

Conclusion:DR, with gold nanoparticles not only minimized the required amount for the purpose of inducing cell death.but also maximizing their efficiency and quality.

Keywords: Gold nanoparticles , Death receptor, DR5, Mouse antibodies

1. Introduction

The developments of nontoxic and biocompatible magnetic particles have been clarified for biological applications since mid-1980s [1]. Magnetic particles have great features as high performance biomaterial used for transport and separation of cells [2], MRI [3], hyperthermia [4] and drug delivery [5]. Nowadays, nanocarriers encounter numerous barrier enrooted to their target, such as mucosal barriers and non-specific uptake [6,7].

Tumors have some general features like leaky blood vessels and poor lymphatic drainage. Free drugs may diffuse nonspecifically and nanocarriers can extravasate into the tumor tissue via leaky vessels by EPR (enhances permeability and retention) effect [8,9]. Experiments performed through using liposomes of different mean size suggest that the threshold vesicle size for escape into tumor

is ~400 nm [11] while other studies have shown that particles with 200 nm diameters are more effective [6, 10, 11]. Nowadays, developments in the field of antibody-conjugated NPs have demonstrated attractive results in both in vitro and in vivo conditions. Even more advanced pharmacotherapy agents can exhibit a very low accumulation into the disease site, and an extensive biodistribution into healthy tissues. As a consequence, long-term pharmacotherapy is characterized by significant inefficacy and severe toxicity. Tumor necrosis factor-related apoptosis-inducing ligand, TRAIL, particularly triggered apoptosis upon engagement by one of its two agonist receptors, DR4 [12] and DR5 [13]. The ligands displays specific antitumor activity against a wide range of tumor cells [14,15] without significant side effects [16]. This extrinsic apoptotic pathway has been targeted by two approaches: recombinant human TRAIL ligands [17,18] or its agonistic antibodies against DR4 and DR5 [19-21]. In contrast, antibodies are cheaper and more acceptable in direct contrast to ligands. In this research, the cell death inducing antibodies were conjugated to gold nanoparticles and used as a potential weapon against MCF7 cancer cells.

2. Materials and Methods

2.1 Peptide Synthesis

A little epitopes from extracellular domain of human DR5 protein with 21 amino acids was produced by peptide synthesizer and after confirmation via GC mass spectrophotometry, they were used for immunization.

2.2 Immunization

Six-week-old BALB/c female mice were immunized by intradermal route, using purified synthetic peptide (350µg). Briefly, mice were immunized in two groups: the first group with peptide and complete Found`s (Razee institute, Iran) adjuvant and the second group received the KLHadjuvant.

There were two different kinds of control groups, non-immunized mice and immunized with pure adjuvants. The immunization was completed by injecting three times boosters with 2 weeks interval of incomplete Freund`s and KLH adjuvant (with an equal volume of peptides). Thirty-five days later, the sera were collected for determining the IgG titers of antibody by enzyme-linked immunosorbent assay (ELISA).

2.3 ELISA

The ELISA assay was completed in ELISA strips. The procedure was started by coating 20µg (per 100µl coating buffer) of recombinant whole DR5 protein (which were produced in our laboratory, under publish); then, they were incubated an over-night in 4^{o}C. The blocking took 60 min incubation with fat-milk. Then, the IgGs were added (dilutions 1:1000 to 1:64000) and incubated for 30 min at 37^{0}C. It was followed by incubation with secondary antibodies (goat derived anti-hen-IgG). HRP was conjugated under the same condition. Washing occurred in every step with PBST for 3 times. At the last step, absorbance of TMB reaction result with HRP was measured at either 450 nm (reactions stopped with 1M hydrochloric acid).

2.4 Nanoparticle Synthesis

1.5 ml of 25 mM aspartic acid solution was added to 2.5 ml of de-ionized water and the mixture was heated till boiling. Upon boiling, 5ml of chloroauric acid solution (such that the molar ratio of aspartic acid to chloroauric acid was adjusted to 7.5) was added to this mixture under vigorous stirring and heating conditions till boiling. After a few minutes, the reduction of the gold salt (Au^{3+}) to GNPs (Au^{0}) was confirmed by the appearance of a dark-red colloidal solution. When the color of the

colloid stabilized, the reaction was rapidly quenched in ice [22].

2.5 Conjugation

By means of electrostatic and hydrophobic binding interactions between GNPs and antibodies, conjugation took place. For preparation of GNPs–antibody conjugates, GNPs (600 μg) were mixed with the antibody solution (300 μg). To complete the process, the mixture was stirred overnight at room temperature.

2.6 UV–Vis Spectrophotometery

The optical properties of the gold colloidal solution (with and without conjugation) were monitored on a Shimadzu dual beam spectrophotometer (Model 1601) in the range of 300–700 nm. Quartz cuvettes with 1 cm optical length were used for all measurements.

2.7 Cell Culture

The MCF7 cell lines (human breast cancer) were grown in RPMI 1640 medium with 10% fetal calf serum (V/V) and 1% penicillin/streptomycin. Cells were incubated at $37^{o}C$ with 5% Co_2.

2.8 MTT Assay

The process began by incubation of 10^4 cells per well in 96-well for 24 hours in $37^{0}C$ and 5% Co_2. In the other day, the medium was removed and the cells were incubated with 100 ml of MTT solution for 2 hours in mentioned conditions in a dark incubator. The supernatant ease was removed and the produced color was resolved in 200 ml acidic isopropanol and was mixed properly. The absorbance of the solutions was read by ELISA Reader (570nm).

3. Results

3.1 Immunization

The small peptides as an antigen essentially need a strong kind of adjuvant to properly induce the immune system of animals. The immunization of mice in this project was executed through two different kinds of adjuvants (first with common adjuvant, Fround`s adjuvant (21A), and second a specific adjuvant for small peptides, KLH one (21K)). The blood sample was collected, with the ELISA results significantly demonstrating the antibody production in mice. Antibodies specifically reacted to the DR5 whole protein which was produced in our laboratory (under publish) (figure 1). It seems that both of adjuvants could induce the immune system properly, though as expected, those mice which were immunized by antigen and KLH adjuvant remarkably displayed better results and obtained antibodies were more significant. In contrast to test group antibodies, the control group’s antibodies (by receiving just Fround`s adjuvant (control-A), just KLH adjuvant (control-K)) and the antibodies from non-immunized mice (control) had no affinity to the target protein (DR5).

Figure 1. ElISA result of produced IgY against extracellular domain of DR5 (21amino acids).ElISA of mice antibody immunized which by antigen and Fround`s adjuvant (21A), with antigen and KLH adjuvant (21K), the golf nanoparticle conjugated of them (21AN and 21KN, sequentially), with just Fround`s adjuvant (control-A) and without injection (control). Numbers in y axis is dilution sequentially (1/1000, 1/2000, 1/4000, 1/8000, 1/16000, 1/32000 and 1/64000). P value of 21, is 0.00041 that are according to $p<0.05$, have sense.

3.2 Nanoparticle Production and Conjugation

At the first step, $HAuCl_4$ salt was reduced by adding the aspartic acid to produce the nanogold-particles. The carboxyl group of aspartic acid interacts with the desired antibody and allows it to make antibody-nanoparticle complex. Figure 2 shows the UV-Vis spectrum of colloidal gold nanoparticle. As seen in figure 2, the gold nanoparticle and the conjugated antibody shows a brand absorption in 530 nm. The shift in top hill of the conjugated antibody confirmed the success of conjugation process.

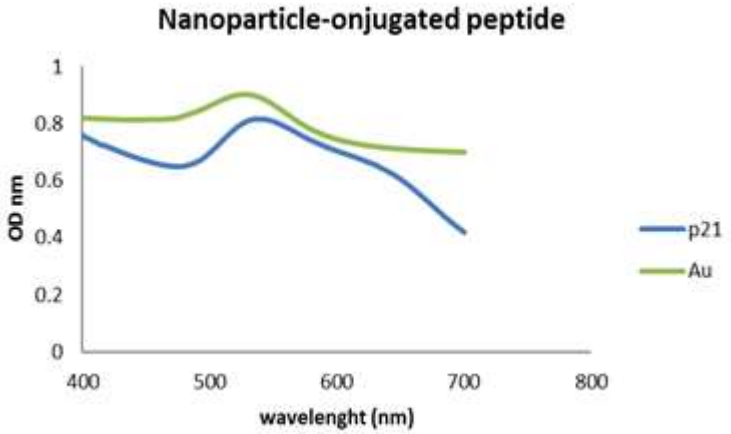

Figure 2. UV-visible spectrum. Colloidal gold nanoparticles (up, green). UV–Vis spectra of antibody-nanoparticle complex (down, blue).

3.3 Anticancer Analysis

Cell death-inducing activity of two kinds of IGGs, gold nanoparticle conjugated (21AN, produced antibody against the peptide injected by Fround`s adjuvant conjugated with gold nanoparticle and 21KN, antibody from immunized hens by antigen and KLH adjuvant conjugated with

gold nanoparticle) and without any manipulations (21A and 21K) were evaluated on MCF7 breast cancer cells. The cells were treated with various concentrations of obtained antibodies; then, 0.25 µg/ml were chosen and allowed to act on cells for 24 hours. The cell viability in the last point was determined by MTT assay. It was not surprising that they could specially kill the cancerous cells, though the better activity of conjugated antibodies was significant (figure 3). The reduction of acquired concentration of antibody in these concentrations had no death effect; hence, they were omitted in figure 3. The significant differences between two kinds of antibodies emphasize their conjugation efficiency with gold nanoparticle. Additionally, among two conjugated IgGs (21AN and 21KN), the second one, as expected, had the best effect on induction death.

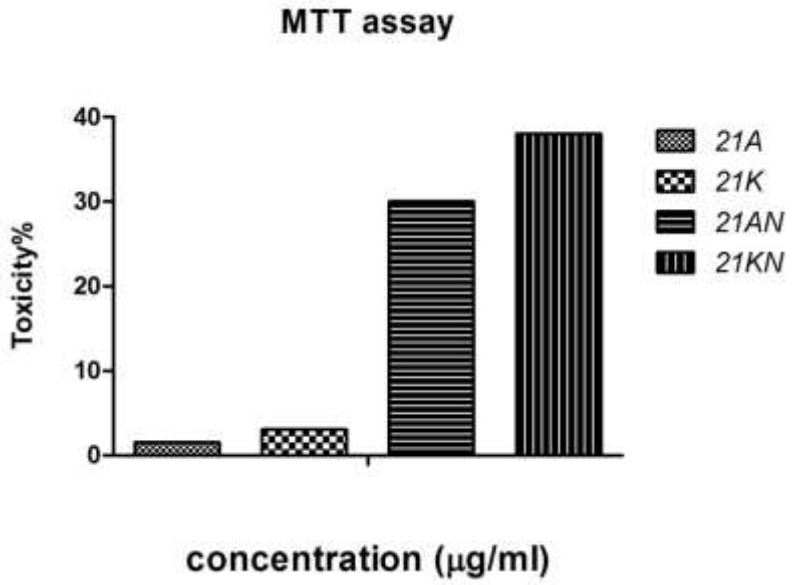

Figure 3. MTT results. (A) Toxicity percentage of IgGs (21A,21K and 21AN, 21KN) on MCF7 cells. The P value is 0.000042 according to P<0.05 has no sense.The percentage of toxicity was calculated BY using the following formula: %toxicity= (AT/AUT – 1)×100, where AT is absorbance of treated cells, and AUT is absorbance of untreated cells.

4. Discussion

In cancer therapy world, researchers are trying to find special agents which could acceptably deliver to the target cells. DR4 and DR5 share significant similarity in the structure of their genes, expression pathways in human body and signaling downstream The mature DR5 receptor contains 411 amino acids, including three cysteine-rich repeats in external domain [23]. DR4 and DR5 showed high identity which was about 66% [12,24-26], but for the present research, DR5 has been chosen.DR5 was described as contributing more than DR4 to the overall apoptotic activity of TRAIL in apoptosis signaling cancer cells [27-29]. The results confirmed such predictions. Hemocyanins act as oxygen-transporting proteins in many arthropod and mollusc species [30]. In addition to this biochemical function, the hemocyanin of the Californian giant keyhole limpet *Megathuracrenulata*, amarine gastropod, is known to be a potent immune activator [31, 32]. Small peptides special adjuvant, KLH, significantly worked better and strongly recommended THATUSE in such immunization. Gold nanoparticles (NPs) could be used for basic diagnostic strategies [33] and therapeutic purposes [34], which are both based on physical characterization of gold NPs.

Furthermore, such particles have extra benefits: they are resistant to oxidative corrosion and are therefore considered stable to the various environments encountered within the human body. Gold would appear safe in in-vivo, though beyond this fact they have demonstrated a significant reduction in cell viability when exposed to high concentrations of gold-based NPs [35]. The amino acids in nanoparticle production challenged its N-terminal with Au and the other free C-terminal group could interact with several kinds of proteins such as antibodies. Attachement of functionalized gold nanoparticles to antibodies makes a stable nanoparticle complex. Special attention is given to recent advances in these engineering approaches: specifically, to target nano-medicines against severe diseases and to the development of multifunctional nano-platforms for combined selective diagnosis and effective pharmacotherapy.

The cell-specific endocytosis of the NP at the site of disease provides an attractive feature for a variety of diagnostic and therapeutic strategies. Furthermore, an additional feature of such kind of NPs mediated delivery is the possibility of inducing multivalent receptor activation to trigger receptor-activated signaling, leading to downstream effects of such apoptotic signaling [36]. In addition, some of these studies have shown that NP size and surface ligand density are referred to as critical key features [37]. According to MTT results, our produced antibodies significantly kill the MCF7 cancer cells. And yet, in contrast to the normal antibodies, the conjugated ones with gold nanoparticle, having huge differences in the same concentration, have the ability to kill the cells. Additionally, the results confirmed our latest experiences of antibodies against 21 aminoacids [38]. This newly produced antibody (by administering the two new adjuvants instead of liposomes) could also recognize the DR5 protein properly and the results proved to be more acceptable. Several researchers introduced different kinds of monoclonal antibodies against DR5 protein [39-43] which are expensive and difficult to obtain. They also poorly induce death in cancer cells and mainly need manipulations in their structure or extra components such as chemotherapy agents to be effective. Immunizing the mice against 21 aminoacid length peptide extracted from the extracellular domain of DR5 protein by injecting with KLH adjuvant in contrast to other kinds of adjuvants (Freund or liposomal constructs) were remarkably more efficient. Significantly, their conjugation to gold nanoparticles reduces the minimum concentrations for inducing death in cancer cells.

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Conflict of interest

The authors declare no conflict of interest.

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