Detection of Anticancer and Apoptotic Effect of the Produced IgYs against the Three Extracellular Domain of Human DR5 Protein

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Abstract

Background: TNFα cytokine family in the body plays divers' roles in the cellular events such as cell proliferation, differentiation, necrosis, septic shock and apoptosis. In response to TNF therapy, several cell signaling pathways activated in cells which in different manners can lead to apoptosis or necrosis. However induction of apoptosis is depended on one of its important members, TRAIL and its receptors that will be followed by apoptosis activity. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and especially the DR5, is generating considerable interests as a possible anticancer therapeutic agent because of its selective activation in apoptosis of this receptor as a superior affinity to ligands.

Methods: The study was performed in invitro assay and the anticancer effects of the produced antibodies were assumed by MTT and flowcytometric methods. In the first step for immunization of the hens, three selective small peptides from extracellular domain of DR5 which were chemically synthesized, injected to hens and after the proper immunization of them, IgYs were extracted from the egg yolk. After assumption of specificity of the purified IgYs against the whole DR5 protein, they were performed in MTT assay and flowcytometric colorimeter.

Results: After confirmation of synthesized peptides they were injected to hens with Fround`s complete adjuvant. With completing the immunization procedure the specificity of purified IgYs were confirmed by ELISA. The antibodies were significantly killed the MCF7 breast cancer cells, but had divers affect (proliferative) on normal hepatocyte cells. Additionally, significantly they induced apoptosis on the cancerous cells in contrast to control cells.

Conclusion: The results clearly demonstrated that the produced IgYs with reduced cost and time managing could remarkably use as an effective anticancer drug.

Keywords: Cancer; IgY; TNF receptor; TRAIL

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Introduction

Balance between cell division and cell death is of almost important for the development and maintenance of multicellular organisms. This disturbance causes abnormality in cells that subsequently proliferation of the cells goes out of control and cancer appears. The death of cancer is the second cause of death. In 1983, researchers revealed that a major problem with cancer therapy was lack of specificity of the treatments for this kind of cells [1]. They predicted that a significant development in treating cancerous cells would be achieved from a class of agents that have a greater degree of specificity for tumor cells [2]. In the last years, researchers have discovered novel agents which specifically target signaling pathways involved in formation, progression or at least prevention of human cancers [3]. Strategically, targeted cancer therapies are emerging from enormous efforts spent investigating basic signaling mechanisms involved in cell growth and cell death pathways. The cell death pathways that have been reported are including apoptosis, mitotic catastrophe, and necrosis. From these pathways, the apoptosis is

the best characterized one, and there are numbers of therapeutic agents that targeting this ways which were currently used in clinical trials [4]. A good definition of programmed cell death is Apoptosis which is "a genetically programmed biochemical process that removes inappropriate cells and maintains tissue homeostasis under physiological and pathological conditions". TRAIL triggered apoptosis upon engagement by one of its two agonist receptors, DR4 [5] and DR5 [6]. This ligands play a specific antitumor activity against a wide range of tumor cells [7, 8] without significant side effects [9]. The binding of TRAIL leads to trimerization of these receptors, recruitment and activation of Fass-associated death domain (FADD) through the interaction with death domain (DD) on the death receptors. Consequently, FADD activates caspase-8 which leading to form of the death inducing signaling complex (DISC). This complex is sufficient to induce activation of one or more effector caspases (-3 or -7) which then act on finding death subunits in apoptosis [10]. At least this extrinsic apoptotic pathway has been targeted by two approaches: recombinant human TRAIL ligands [11] or its agonistic antibodies against DR4 and DR5 [12]. DR4 and DR5 share significant similarity in the structure of their genes, expression pathways in human body and signaling downstream. The mature DR5 have 411 amino acids that include a signal peptide, three cysteine- rich repeats and intracellular domains of DD [13]. More identity of these two receptors, DR4 and DR5, are in cysteine-rich extracellular domain (66%) and death domain (64%) [5]. DR5 was described as a contributor more than DR4 to the overall apoptotic activity of TRAIL in apoptosis signaling cancer cells [14]. Production of agonists of TRAIL like antibodies can lead to activation of these receptors. From two kinds of poly- and monoclonal antibodies, the major problem of monoclonal antibody is that some antigens are weakly or non-immunogenic for animals. Also this technology involves some steps which cause distress to animals from the immunization itself to collecting of blood samples and even bleeding, which are a prerequisite for antibody preparation [15]. During the past 20 years, the use of chickens against mammals for antibody production has been increased. A major advantage of using birds is the ability of antibodies to be extracted from the egg yolk instead of serum, thus they do not hurt animals. Additionally, antibody productivity of an egg-laying hen is much greater than that a similar size in mammals. Actually hens are recognized as a convenient and inexpensive source of antibodies [16]. From an animal welfare point of view, chickens are an attractive alternative to mammals as antibody producers because large quantities of antibodies can be produced from the egg yolk making restraint from the blood sampling obsolete techniques to the benefit of the animals used for this purpose [17]. In this research we combined DR5 special apoptotic pathway activation by egg yolk antibody (IgY) advantages and hoped that this kind of antibodies could be targeted weapon against cancer cells.

Materials and Methods

Peptide production

The target was chosen from extracellular domain of human DR5 protein and three small peptides with 27, 21 and 15 amino acids which were produced in peptide synthesis center of National Institute of Genetic Engineering & Biotechnology, Iran, by peptide synthesizer and chemical method.

Immunization of hens

Immunization of hens was performed in animal house of Pasteur institute (Tehran, Iran) with a total 350 µg of each peptide plus equal volume of Fround adjuvant per animal by intradermal injection in multiple sites of breath, followed by three subsequent boosters.

ELISA

The ELISA assay was done in SLISA strips. The procedure were started by coating 20µg (per 100µl coating buffer) of recombinant whole DR5 protein (that were produced in our laboratory, under publish) then they were incubated an over-night in 4^{o}C. The blocking was done by 60 min incubation with fat-milk. Then the IgYs were added (dilutions 1:100 to 1:6400 and incubated for 30 min at 37°C. it was followed by incubation with secondary antibodies (goat derived anti-hen-IgY), HRP conjugated in the same condition. After washing with PBST, in final step, absorbance of TMB reaction that resulted by HRP, was measured at either 450 nm (reactions stopped with 1M hydrochloric acid).

Isolation and purification of IgY

Solation of IgY was carried out by separating the egg yolk from the white part. The egg yolk was added two volumes of PBS buffer (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.46mM KH_2PO_4, pH=7.4). Then the solution was mixed with

magnetic stirrer. After that, we added two volume of chloroform and incubate in 4°C for 20 minutes and Centrifuge the solution in 14000×g, for 20 minute, collected the supernatant and discard the plate. 12% (w/v) poly ethylene glycol (PEG6000) was added to the supernatant and mixed with magnetic stirrer for 20 minutes to remove lipoproteins. The mixture was centrifuged at 14000 ×g, 10 minute at 4°C and the supernatant was discard and sediment resolved in PBS (pH 7.4). Finally, the sediment was resuspended in equal volume of phosphate buffer and preserved at 4°C until further use.

SDS-PAGE analysis of IgY

SDS-PAGE analysis was carried out according to the method of Laemmli. Breifly, 15% PAGE was made using Bio-Rad Mini Protein system. 20 µl of test IgY and 20 µl of IgY isolated and purified from immunized and non-immunized hens were resolved at sample buffer and heated at 100°C for 10 minutes. 20 µl from each sample was loaded into each well. Pre-stained protein molecular marker (Fermentas) was used as a standard molecular weight marker.

MTT assay

In this experiment 10^4 cells (MCF7, 3T3) were coated per well in the first day and incubate it for 24 hours. In the second day, media was carefully substituted with antibodies and new medium (final volume should be 200 µl) and the cells were treated for 24 hours at 37°C, 5% CO2. Then supernatant was discarded and added 100 µl of 5 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to each well. The cells were incubated for 1 hour at the same condition in culture incubator. At last carefully remove the media and resolve the color in MTT solvent (isopropanol). Finally, absorbance was read at 590 nm. Then the data was calculated by Pharm software.

Flowcytometric assay

The 1×10^6 cells from MCF7 cancer cell treated with calculated concentrations (IC50s) and incubated at 37°C, 5% Co2 for 3 hours. The cells were extracted by centrifugation (8500×g, 5 min). The centrifuge operation was repeated following wash out with PBS. Flowcytometry analysis was followed by adding citrate phosphate buffer and PI/RNase A staining (Roche) kit according to the kit protocol. After 20 min keeping in last dark room, they were analyzed by flowcytometer immediately.

Results

The egg yolk antibodies extracted with PEG

In several different extraction methods, first of all we should remove the pigments and lipoproteins and finally purify the IgYs. Among several IgY purification protocols, extraction with PEG was chosen. IgY yield and purification quality in this method was considerably comparable with other one`s (not publish). According to figure 1, that shows SDS-PAGE results, IgY proteins with 25-65 KDa weight were produced and amplified in egg yolk. In contrast with control line which contains non-immunized yolk`s antibody, in the test lines, the protein variety reduced and the proteins were almost limited to just IgYs. Obviously, purity of the purified antibody is significant. According to the loaded concentrations which were diluted 1:5 in sample buffer with final 0.008 mg/ml the results was really shining.

Hens successfully immunized and produced significant amount of specific IgY against three small peptides of DR5

The purified IgYs should be analyzed and were examined to identify their specificity. For this purpose, the obtained antibodies were performed in ELISA assay. As you can see in figure 2, they were not only notably attached to the whole protein but also their production amount was remarkable. The control antibodies, antibody obtained from non-

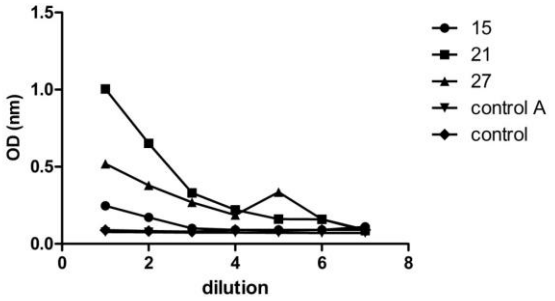

Figure 1. ELISA result of IgY that produced against 15, 21, 27 amino acids of the extracellular domain of DR5 protein; ELISA of three kinds of antibody that extracted from the egg which were with immunized by amino acids (15, 21, 27), with just Fround adjuvant (control A) and without injection (control). Numbers in y axis is dilution respectively (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400). P value of 21, is 0.002 that are according to $p<0.05$, have sense.

immunized hens and hens immunized just with adjuvant, could hardly recognize the DR5 protein. The most scale of antibody which was produced against 21 amino acids peptide (21A) and the least production were in hens that immunized with 15 amino acids peptide (15A). The antibody that was obtained against 27 amino acid (27A), was in middle level of two other antibodies.

IgY that produced against three peptides of DR5 can kill specially cancer cell.

MTT assay is a specific technique for analyzing the death effect of agents. MCF7 cells, breast cancer cell line, were treated with certain amount of antibodies, three test and decontrols. According to figure 3a, three test antibodies significantly in contrast to control one, induce death in cancerous cells. Among the antibodies, 21A has the most effect and two others (15A and 27A) respectively occupy the other two and three steps. The control antibodies not only had no death effect but also induce proliferation in cancer cells. In the second part of the figure 3 (3b), MTT assay results of 3T3 cell line, normal hepatocyte cells are represented. All kind of antibodies in this experiment significantly induce proliferation. 21A antibody has the least and 15A one has the most productivity effect. It seems that efficiency of the antibodies apart from the kind of death or life induction walk in the same way. In table 1, the IC50 concentrations that were calculated by Pharm software is shown. Indeed, the calculated concentrations and the amount of death effects of the antibodies were in the same directions.

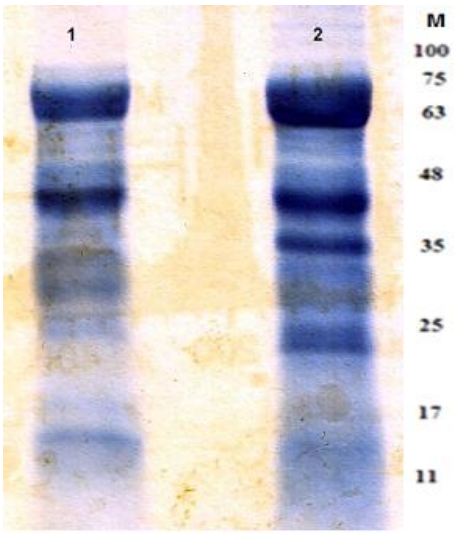

Figure 2. SDS-PAGE (15%) analysis of purified IgY; M: Molecular weight marker, Lane 1: IgY from egg yolk of non-immunized hens, Lane 2: IgY from egg yolk of immunized hens under non-reducing conditions.

Produced IgY induces apoptotic pathways in the breath cancer cells.

Flowcytometric assay and their special colors could help to identify the kind of death induced by different agents. Our produced antibodies significantly induced apoptosis. The results of flowsytometric assay in figure 4 that covered with PI/RNase A demonstrated the apoptosis action. Results in three treatments were not remarkably different. The apoptosis percentage in three different kinds of IgYs varied between 80-89% with no huge fluctuations.

Discussion

The researcher find out that many oncogenes and suppressor genes like p53 which is participate as a guard in native cells which were caused mutations (over activation) or deletions almost in 50% of human cancers. In the other word, they refuse their crucial job, starting apoptosis [18]. So, in this manner cell loses one of the most important key initiators of apoptosis in the cancerous cells. Recombinant soluble of human TRAIL is a candidate for clinical investigation in mice, none-human primates and human [19], cancer therapy because it induces apoptosis in a broad spectrum of cancer cell lines but not in many normal cells, and exhibits potent anti-tumor activity without normal tissue toxicity in various cancer xenograft models [20]. Rieger et al. and Wu et al. use TRAIL and it`s in vitro interaction with the DR5 protein on glioma cells and induce apoptosis in them [21, 22].

Local injection of TRAIL exerted strong antitumor activity on intracranial human malignant glioma xenograft in athymic mice in absence of neurotoxicity. From the sequence alignment of the

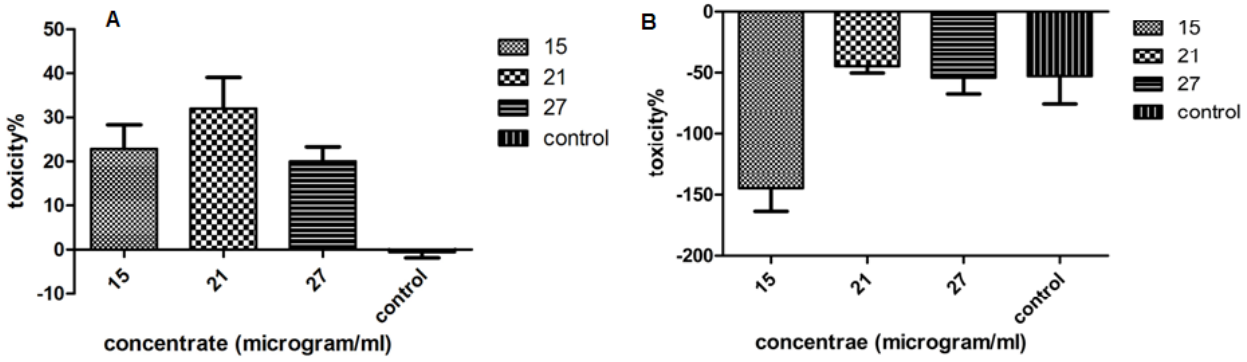

Figure 3. MTT results; (A) Toxicity percentage of IgYs, control A and control, on MCF7 cells. The P value is 0.0006 according to $p<0.05$ it has no sense. (B) The percentage of toxicity of mentioned antibodies on 3T3 cells. P value is 0.0045 according to $p<0.05$ it has no sense. The percentage of toxicity was calculated using the following formula: toxicity%= (AT/AUT– 1) ×100, where AT is absorbance of treated cells, and AUT is absorbance of untreated cells.

Table 1. IC50 dosage of antibodies that induce death on MCF7 cells; Dosage mg/ml) for killing 50% of MCF7 cell with IgY-15, -21, -27 and control A. The results assessed by following formula: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X))).

antibody	IgY-15	IgY-21	IgY-27	Control-A	control
IC50 (µg/ml)	536.807	282.389	637.373	2017.8	5142.13

different TRAIL receptors [23] it is observed that, the receptor cystein rich domains (CRDs) involved in the extraction with TRAIL (CRD2 and CRD3) are highly conserved. In DR5, there seems to be three cystein rich repeat [24], which in extracellular domain [25] are necessary for attaching to ligand and then activate the receptor. In this point, three peptides with special possible characterize were chosen. All three peptides have cystein and in the other hand have their efficient conformation. TRAIL-mimicking antibody, which binds to receptors and activated it, can be a good substitute therapy. Nowadays one kind of this antibody against DR4 for treatment of hepatological malignancies is in phase I clinical trial [26]. Additionally, production of antibody against these little peptides has two advantages. In one side, we obtain one kind of polyclonal antibody which has limited variety (because of limited number of antigens represented on MHCs) and in the other hand restriction of the area that antibody should recognize could cause specify of such kind of antibody against the whole protein. The serum concentration of IgY, IgA, and IgM has been reported to be 5.0, 1.25 and 0.61 mg/ml, respectively [27]. According to described portions, IgY makes uptake 75% of the total immunoglobulin pool. Antibodies are transferred from hen to chicken via the latent stage of the egg

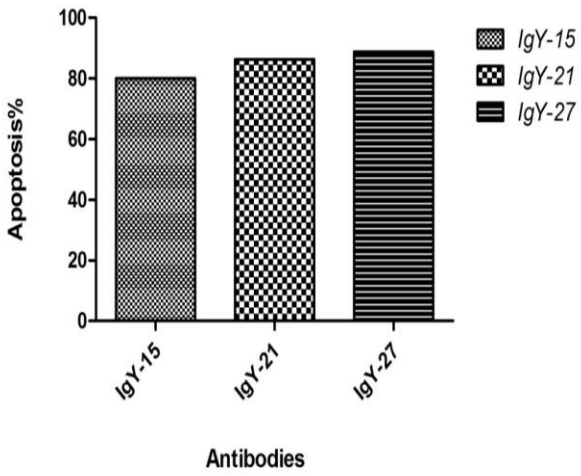

Figure 4. Flowcytometric results. The apoptotic percentage induction of IGYs on MCF7 breast cancer cells.

and play an important role in immunological function for the relatively immune incompetent chicken to resist various infection diseases [20].

Serum IgY is selectively transferred from the hen`s circulatory system across the oolemma into the maturing oocyte in the ovarian follicle [28] and significantly it makes extraction and purification easier and more inexpensive. As we expected, the whole scale of antibody production in contrast to injection of bigger protein is considerable. It is obvious, moreover the reduction of using peptide will be increase the possibility of induction of immune system. 21A peptide had ideal scale to uptake in APC cells and induce B-cell antibody production machine. It is expected that purified antibodies in such kind of immunization has the best and efficient interaction with its antigen/receptor and at the end point could lead to best activation form and so the best answer or death inducing in the responsible cells. In the control hens as we expected, there were no special antibodies and the other kind of antibodies that rationally were produced in hens' body 93% of whole IgYs did not have any reaction with our peptides or with the receptor on our desire cells. Researchers introduce several kinds of specially monoclonal antibodies against DR4 and DR5 for treatment of different kind of malignancies and they were in clinical trials [29-33].

All such antibodies, unfortunately, could not kill cancerous cells lonely and need the association of chemotherapical treatments. Additionally, the IgY-21 not only in MTT results on MCF7 cells showed the most dead induction but also on 3T3 cells in the same manner had the most proliferative induction. As we noticed, the several different kinds of receptors show diverse responses to TNFα induction. DR5 receptors with 80% accumulation on cancer cells could specially initiate apoptosis pathway and the other kinds of TNFα receptors on the normal cells in the opposite pathway not only had no death effect but also initiated proliferative pathways. The flowcytometric results specify the death kinds, apoptosis. Interestingly, the predicted death was almost completely apoptosis. Furthermore, we could surely introduce our IgYs as an apoptotic inducing weapon against the cancer.

Conclusion

The results clearly demonstrated that the produced IgYs with reduced cost and time managing could remarkably use as an effective anticancer drug.

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Conflicts of Interest

There is no conflict of interest in this article.

Authors' Contribution

Shaghayegh: performed laboratory operation, Manuscript preparation. *Mehrdad Hashemi*: Design of the study, provided technical support and data analysis and revised the paper extensively.

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