

RESEARCH ARTICLE

Apoptotic Killing of Breast Cancer Cells by IgYs Produced Against a Small 21 Aminoacid Epitope of the Human TRAIL-2 Receptor**Shaghayegh Amirijavid¹, Maliheh Entezari¹, Abolfazl Movafagh², Mehrdad Hashemi^{1*}, Alireza Mosavi-Jarahi⁴, Hossein Dehghani^{3*}****Abstract**

TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand belongs to one of important cytokine superfamilies, tumor necrosis factor (TNF α). TRAIL-2 receptor agonists activate several cell signaling pathways in cells in different manners and could lead to apoptosis or necrosis. Agonistic egg yolk antibodies like IgY which have been developed in a selective manner could activate TRAIL death receptors such as TRAIL-2 (DR5) and thus apoptosis signaling. We here investigated induction of apoptosis in human breast cancer cells (MCF7 cell line) by an IgY produced against an 21 aminoacid epitope of the human TRAIL-2 receptor. As the first step a small peptide of 21 aminoacids chosen from the extracellular domain of DR5 protein was produced with a peptide synthesizer. After control assays and confirmation of the correct amino acid sequence, it was injected to hens immunized to achieve high affinity IgYs. At the next step, the produced IgYs were extracted and examined for specificity against DR5 protein by ELISA assay. Subsequently, the anticancer effect of such IgYs was determined by MTT assay in the MCF7 human breast cancer cell line. The produced peptides successfully immunized hens and the produced antibodies which accumulated in egg yolk specifically recognized the DR5 protein. IgYs exerted significant toxicity and killed MCF7 cells as shown by MTT assay.

Keywords: Breast cancer - MCF7 cell line - IgY - chicken yolk immunoglobulin - DR5*Asian Pac J Cancer Prev, 17, Cancer Control in Western Asia Special Issue, 293-297***Introduction**

Cancer is a disease of a genome and normality of cell proliferation. So in these types of cells, several cycles of duplication without right replication cause amplification of mutations. Apoptosis or cell programmed death is an intrinsic death pathway which is used to regulate cell's many hemostatic processes. Occupying the death receptors from TNF α superfamily such as CD95 or TRAIL receptors by their specific ligands can recruit the subsequent effector molecules like caspase 8 which will lead to performing complex death inducing signal complex (DISC) (Ashkenazi, 2008). In the next step, it will activate, or alternatively permeabilize mitochondrial membrane by cleaving Bid and releasing the cytochrome c out of it. At least, both of these two pathways cause apoptosis in cells. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand is currently attracting great interest as a potential anticancer drug. TRAIL, selectively induces apoptosis in cancerous cells by their specialized death inducing receptors.

TRAIL has five different kind of receptors: DR4

(TRAIL-R1) and DR5 (TRAIL-R2) and the decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG) (LeBlance and Ashkenazi, 2003; Kischkel et al., 2000; Sprick et al., 2000; Bodmer et al., 2000; Kimberley and Screaton, 2004). However, DR4 or DR5 receptor-specific TRAIL variants would permit new and tumor selective therapies. The researcher indicated that DR5 contributes more than DR4 to the overall apoptotic activity of TRAIL in apoptosis signaling of cancer cells (Kelley et al., 2005; Almasan and Ashkenazi, 2003; Ichikawa et al., 2001). There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons (Bollen and Hau, 1996; Svendsen et al., 1994; Tini et al., 2002). The egg yolk antibodies (IgY) in recent years remarkably used for scientific (Schade et al., 1997), diagnostic (Di Lonardo et al., 2001), prophylactic (Almeida et al., 1998; Sarker et al., 2001) and therapeutic purposes (Lemamy et al., 1999), and on the other hand for veterinarian therapy against bacteria such as enteropathogenic *Escherichia coli* (Amaral and Franco, 2002). Several advantages of IgY like, the phylogenetic distance between birds and

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mammals, help to increase immune response in host body (birds) in comparison to mammalian hosts (Leslie and Clem, 1969; Hau et al., 1980; Hau et al., 1981). The combination of this two advantages, occupying the death receptor with an antibody that produced in birds, can give a strong weapon against MCF7 cancerous cells. In this study we choosed a small part of the extracellular domain of the DR5-receptor, synthesized it and used for immunization of the hens. At least, we evaluated its effect on breast cancer cells.

Material and Methods

Peptide production

A part of the whole protein that contains 21 amino acids (SCKYGQDYSTHWNDLLFCLRC) is produced in peptide synthesis center of National Institute of Genetic Engineering & Biotechnology, Iran.

2.2. Immunization of hens

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

ELISA

Ninety-six-well ELISA plates were coated with 20µg of either recombinant synthesized peptide and were incubated coating buffer for one over-night in 4oC. The coating was followed by 60 min incubation with blocking buffer then incubated for 45 min with the tested IgY antibody (dilutions 1:100 to 1:1600 were used), then 30 min incubation with secondary antibodies (goat derived anti-hen-IgG), HRP conjugated. We washed the wells in every step for 3 times with PBST (137mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.46 mM KH2PO4, 0.05% Tween-20). In the detection step, citrate buffer plus OPD and hydroxide peroxide as a catalyzer were added and absorbance was measured at either 492 nm.

Isolation and purification of IgY

Isolation of IgY was carried out by separation of the egg yolk from the white part. The egg yolk was added

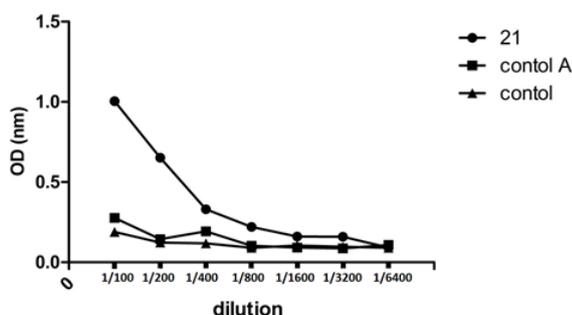


Figure 1. ELISA Result of IgY that Produced Against 21 Amino Acids of the Extracellular Domain of DR5 Protein (IgY-21). ELISA of three kinds of antibody that extracted from the egg with immunization with 21 amino acids (21), with just Fround adjuvant (control A) and without injection (control). P value of 21, is 0.05 that are according to $p < 0.05$, have sense.

two volumes of phosphate buffer (sodium phosphate 0.1M, NaCl 10mM, pH=7). To ensure the hemogenising, they were mixed with magnetic stirrer. Then, we added two volume chloroform and incubate in 40C for 20 minutes. The solution was centrifuged in 14,000 ×g, for 20 minutes and the supernatant was collected and the plate was discarded. 12% (w/v) polyethylene glycol (PEG6,000) was added to the supernatant and mixed with magnetic stirrer for 20 minutes to remove lipoproteins. The mixture was centrifuged at 14,000 ×g, 10 minutes at 4oC and the supernatant was discarded and sediment was resolved in PBS (PH=7.4). For more purification of IgY the last step was repeated for three times. At least, the sediment was resuspended in an equal volume of phosphate buffer and preserved at 4oC until further use.

SDS-PAGE analysis of IgY

Sodium dodecyl sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli. 9% PAGE was made using Bio-Rad Mini Protein system. 20µl of IgY-21 and 20µl of IgY which was isolated and purified from non-immunized hens were separately diluted in 1:2 ratio with sample buffer (62.6 mM Tris-HCl, pH 6.8, 25% glycerol [v/v], 2% SDS [v/v], 5% β-mercaptoethanol) and heated at 100 oC for 5 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

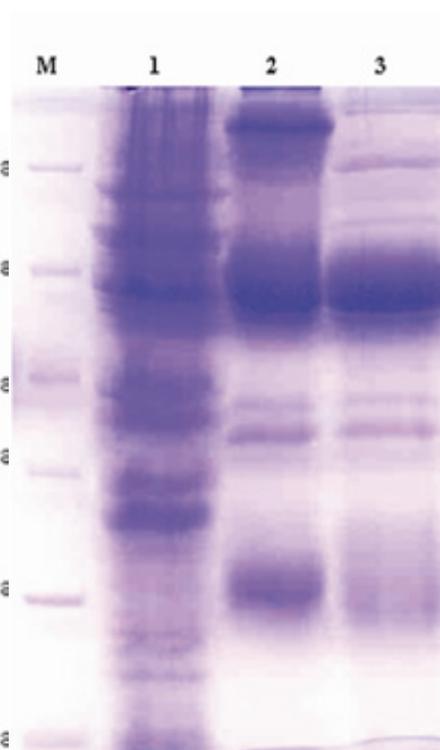


Figure 2. SDS-PAGE (9%) Analysis of Purified IgY. M, Molecular weight marker; Lane 1, IgY from egg yolk of non-immunized hens; Lane 2, IgY from egg yolk of immunized hens under non-reducing conditions; Lane 3, IgY from egg yolk of immunized hens under reducing conditions.

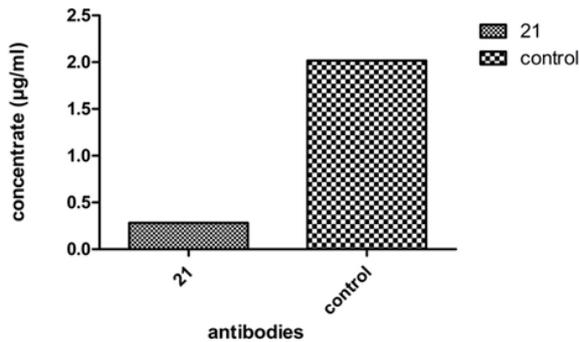


Figure 3. IC50 Dosage of Antibody that Effects on MCF7 Cells. Dosage for killing 50% of MCF7 cell with IgY-21 (0.07 µg/ml) and control A (2.02 µg/ml); The results assessed with Pharms software.

MTT assay (a colorimetric assay) detects the reduction of MTT (Sigma) by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and hence the measurement of cytotoxicity of cell and viability. Briefly, 104 cells/well were treated with various concentrations of antibodies (final volume should be 200 µl). After 24 hour incubation the cells were washed twice with phosphate buffered saline (PBS) and MTT (0.5 mg/mL PBS) was added to each well and incubated at 37°C for 3h. The formazan crystals that formed were dissolved by adding dimethyl sulfoxide (100 µL/well), and the absorbance was read at 570 nm using a microplate scanning spectrophotometer (ELISA reader, Organon Teknika, Netherlands). Toxicity level was calculated by the following formula:

$$\text{Cytotoxicity \%} = 1 - \frac{\text{Mean absorbance } \bar{f} \text{ toxicant}}{\text{Mean absorbance } \bar{f} \text{ negative control}} \times 100$$

$$\text{Viability \%} = 100 - \text{Cytotoxicity \%}$$

To diminish test error level, MTT strain was added to some wells without cells and along with other wells, absorbance level was read and ultimately subtracted from whole the absorbance.

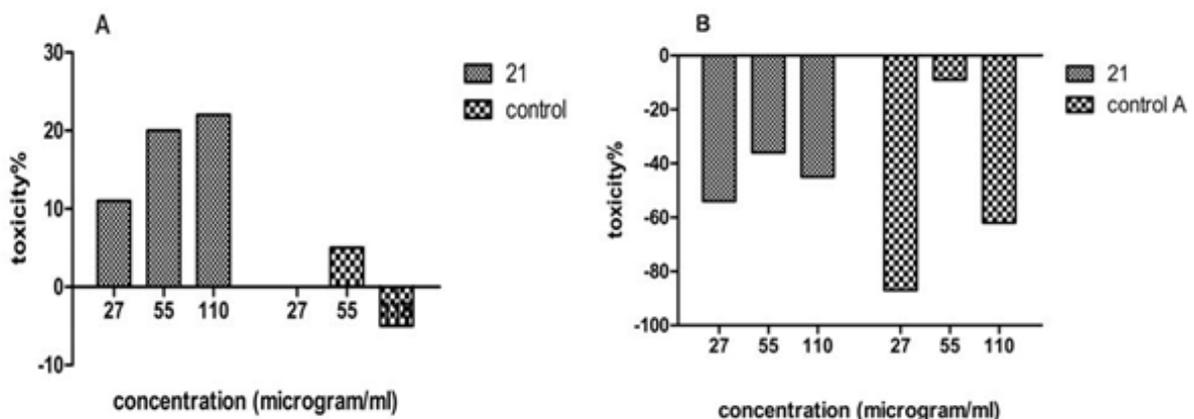


Figure 4. MTT Results (A), Toxicity percentage of IgYs of 21, control A and control, on MCF7 cells; The P- value is 0.02 according to $P < 0.05$ it has no sense; (B), The percentage of toxicity of mentioned antibodies on 3T3 cells; P- value is 0.76 according to $P < 0.05$ it has no sense; The percentage of toxicity was calculated using the following formula: %toxicity= $(AT/AUT - 1) \times 100$, where AT is absorbance of treated cells, and AUT is absorbance of untreated cells.

Flowcytometry analysis

1x10⁶ cells from MCF7 cancer cell line were incubated under treatment of 0.067 µg/ml of antibodies that produced against 21 amino acids of DR5 protein (mentioned previously). As well as control cells, the treated cells were centrifuged after incubation and they were performed in interval periods of 6 hours. The centrifuge operation was repeated following wash out with PBS. Flowcytometry analysis was accomplished by using Annexin-V-fluor staining (Roche) kit. Accordingly after centrifuge, 500 µl of Annexin-V-fluor binding solution and 5 µl from Annexin-V-fluorecein and propidium iodide were added to the cells followed by the cells were incubated in 15-25°C for 5 minutes. Lastly, they were analysed by flowcytometer immediately after incubation.

Results

Efficient production of IgY against little epitope of DR5

To test the production of antibody after immunization period, we performed the ELISA assay with IgY samples. Egg yolk from immunized hens were used for IgY purification. After purification of IgY from egg yolk, we demonstrated that there was a significant amount of antibody in the yolk of the hens. The results shown in figure 1. As indicated, immunization with 350 µg of peptide, efficiently induced antibody secretion. Actually, the produced IgY (IgY-21) in blood at least transferred into yolk and concentrated there. As you can see in Figure 1, in contrast to control IgYs, test's IgY proprietary recognizes DR5 epitope. Control IgYs, the antibodies produced against just Freund's adjuvant and the antibodies obtained from nonimmunized hens, had no affinity to our peptide. It was happily confirmed the specificity of IgY-21 against DR5 protein. The purification was assessed using SDS-PAGE under reducing and non-reducing conditions (Figure 2).

IgY-21 kill cancerous cells selectively.

The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic agents

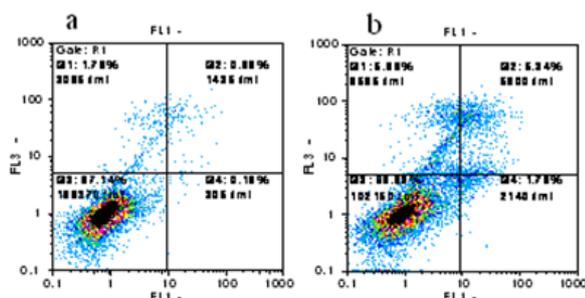


Figure 5. A, Untreated control; B, Incubated with IgY-21; MCF7, human breast cancer cell lines labelled with annexin v and PI; Q2, necrotic cells and cells probably in second phase of apoptosis; Q3, apoptotic cells; Q4, live cells.

leads to apoptosis or necrosis, it measures the increasing of cell toxicity. To estimate the ability of IgY-21 to kill the cancerous cells, we used the MTT assay. The result showed half of the cells within 0.067 $\mu\text{g/ml}$ of IgY-21 survived (Figure 3). We expected such results according to the specific interaction of IgY-21 with DR5 receptor, it seems that IgY can induce initiation of death cascade in such cells (Figure 4A). It is obvious that IgY-21 efficiently could kill the MCF7 cancerous cell though this dosage does not have any effect on normal mice hepatocyte cells (3T3 cell line) (Figure 4B).

The death that IgY-21 induced is definitely apoptosis.

Annexin V/PI assay significantly indicates that the attachment of antibody to its receptor start the apoptotic pathway in cancerous cells. In Figure 5, flow cytometric results indicated apoptotic bodies. In treatment for just 3 hours 8.2% of treated MCF7 cells in contrast to untreated cells had apoptosis (primary and secondary level of apoptosis).

Discussion

Nowadays, the researches in wild field of apoptosis due to expanding our knowledge about its special role in clinical trail against tumours. Apoptosis is an energy-dependent process that can actively decrease the improperly developed cells or the cells which have genetic damages (Thompson, 1995). Many oncogenes and suppressor genes that participate in the human cancer pathophysiology, such as Ras and p53, represent overactive or deleted in human cancers. P53 acts such as a guard in native cells and which upon induction of DNA damage is pivotal in the DNA repair process (Lebedeva et al., 2003). p53 is inactivated in approximately 50% of human cancers, representing the most common genetic aberration in human malignancy. So, as mentioned cell loses one of the most important key initiators of apoptosis in the cancerous cells. As we wanted to start this process try to find another properly substitute for it. It seems death receptors are a good one. TRAIL is a 32 kDa protein which is expressed in a many cells of normal fetal and adult tissues. Such observations demonstrated that in normal cells there is no toxic effect. The researchers indicated that TRAIL can initiate apoptosis in transformed and malignant

cells, but not in normal cells (Lebedeva et al., 2003). The selective nature of the anticancer activity of TRAIL was demonstrated by studies in mice and non-human primates (Ashkenazi, 1995; Walczak et al., 1999). But the reports that indicated the cytotoxic effect of this on normal cells (Jo et al., 2000; Ozoren et al., 2000), suggest the non optimization of this kind of induction (Lawrence et al., 2001). TRAIL-mimicking antibody which is bound to receptor and activate it, can be a good substitute therapy. Nowadays several kind of this antibodies against DR4 and DR5 for treatment of different kind of malignancies are in clinical trials (Salcedo et al., 2002; Chen et al., 2012; gue et al., 2005; Xiang et al., 2013; Du et al., 2011). Though unfortunately, almost all of them could not kill cancerous cells lonely and need the association of chemotraceutical treatments.

After Klemperer discovery that the immunization of hens results in transferring the specific IgY from serum to eggs till now, the scientifics produce several kinds of IgYs that can stop or control their target pathological organisms (Chalghoumi et al., 2009; Dias et al., 2010). Beside other benefits of IgYs, chicken antibodies do not activate the mammalian complement system nor interact with rheumatoid factors, or bacterial and human Fc receptors. It seems using the antibody which produced by hens to attaching this receptor and mimicking its work can be efficient. As we see, the antibody against a small part of the extracellular domain of DR5 receptor could specifically attach and induce its activation.

The 21 amino acid peptide, have cysteine rich repeats that play an important role in the extracellular domain of DR5 receptor. This part plays a special role in the efficient folding of the ligand attachment domain. Furthermore, according to the results we obtained, it seems that occupying this part with the IgY-21, could definitely activate the receptor. Our peptide was small and it couldn't properly stimulate immune response in hens. So the antibodies yields reduced. It would be because of less uptake of peptides with APC cells or presence of less APC cells under dermal parts. But, such little amount when concentrated in egg yolk, we could obtain more antibodies. IgY-21 with high specificity attach the DR5 receptor on cancerous cells and induce death. The flow cytometric results demonstrated that the IgY-21 definitely cause apoptotic death in MCF7 cells. In contrast, this IgY had no cytotoxic effect on hepatocyte cells (3T3), even in this concentration increased cell proliferations. These two interesting results reveal the specificity of IgY-21 to killing cancer cells versus normal cells and indicated that the death was targeted to cancer cells with reduced side-effects, apoptotic pathways.

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