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EXPERIMENTAL STUDY

Generation and characterization of chicken egg yolk antibodies (IgY) against TNFR1

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Abstract: TNF is from a big family of cytokines with different activities in different parts of the body. Among the various activities of TNFR1, induction of apoptosis by a receptor appears to be an attractive and promising one. This can be achieved through the death domain of the receptor in cells that are stimulated by ligand, to induce apoptosis. Activation of the receptor occurs through its occupation by ligands or its antagonists such as antibodies. Several kinds of antibodies, including antibodies of mammals and birds are used in the research and therapy field. Avian antibodies are highly regarded which is due to the many positive characteristics they have. Firstly, total protein of TNFR1 was cloned. Blood sampling was performed, white blood cell separation, extraction of RNA and at cDNA synthesis. After making sure from synthesis of cDNA, it was used as template for PCR reaction. The cloned fragment in the prokaryotic expression vector, pET28a, transferred to prokaryotic host, BL21(DE3) and the protein (TNFR1) expressed. After protein purification by affinity column were injected to immunize the chickens. Interestingly, antibodies purified from egg yolk of immunized chickens, in ELISA assay showed sufficient specificity. Such antibodies could able to ensure quick and immediate protection against several biotargets *(Fig. 4, Ref. 37)*. Text in PDF *www.elis.sk.*
Key words: IgY, TNFR1, cloning.

Introduction

Tumor necrosis factor α (TNF) is a kind of cytokine which has pleiotropic functions in inflammation, immunity, in several steps of cell proliferation control, differentiation and apoptosis (Ashkenazi and Dixit, 1998, Wallach et al, 1999, Forin-Mleczek et al, 2002). TNF family still has a growing number of cytokines like TNF, Fas ligand, lymphotoxin α, CD40 ligand and TNF-related apoptosis-inducing ligand (TRAIL). Like most other members of the TNF ligand family, TNF, is also a transmembrane heteroterimeric protein (Rauert et al, 2011). Generally, TNF α can initiate proapoptotic signaling cascade which is mediated by its two receptors, TNFR1 and TNFR2. Like other receptors of the TNF α family, TNFR1 and TNFR2 are proteins with extracellular, transmembrane and cytoplasmic domains. The two receptors have similar extracellular sequences that are rich in cysteine and that is the hallmark of the TNF α`s big family. Besides similarity of these two receptors, TNFR1 alone has a cytoplasmic death domain (DD) with an 80 aminoacids, which can rapidly initiate the apoptotic signaling pathway in the cells. In contrast to TNFR1, TNFR2 does not have a death domain and also does not have a structural or functional resemblance to that of TNFR1 (Faustman et al, 2010, Tartaglia et al, 1993, Hsu et al, 1995). TNF α attachment to TNFR1, starts apoptosis (Gon et al, 1996). Researchers demonstrated that between two TNFα receptors which are activated by membrane, TNFα in contrast to soluble TNFα remarkably stimulates TNFR1 (Wajant, 2003).

After attachment of the TNFα ligand to TNFR1, it recuits the adaptor protein TRADD, clustering that and as a result they activate death receptor apoptotic pathway (Geering et al, 2011). Though there is a second pathway which relies on TNFR1`s recruitment of the intracellular adaptor proteins, receptor interacting protein (RIP or RIPKI) and FADD, to coordinate downstream signaling by the caspase cascade (Dewson and Kluck, 2009). Then the second step is activation of caspase 8 and other functional caspases like caspase 3, -9 which at least due to functional action of apoptosis (Dewson and Kluck, 2009, Riedl and Shi, 2004, Kroemer et al, 2007).

Eggs are konown for a long time as a food source and a storage place for large amounts of egg yolk antibodies. Immunoglobulin Y (IgY) is a kind of functional mammalian immunoglobulin G (IgG) in birds. These antibodies were transferred to the yolk and they could protect the development of the chick (Karacs et al, 2012). During the evolution of egg yolk, IgY from serum is selectively transferred to the egg yolk via attachment to the receptors on the surface of the yolk membrane (Marrison et al, 2002, Tesar et al, 2008). The other two types of hens body`s immunoglobulins IgA, and IgM accumulated only in the egg whites (Rose et al, 1974). Due to differences between mammalian IgG and IgY in immunological reactions, egg yolk antibodies in many of the works or the

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discovery of biomarkers for diagnosis or treatment are employed. However, most researchers have focused on the use of IgY in passive immunization. This type of antibody has many benefits, such as its large quantities and inexpensive production, non-invasive method for both immunization and to obtaining antibody, no cross-reactivity in the mammalian body and… (Kovacs-Nolan and Mine, 2012), beside this fact that there is a growing field of bacterial resistance, created them a desirable option compared to other types of antibodies (Jenifer et al, 2012). Also, such antibodies could be able to have quick and immediate protection against several bioattacks, like bioterrorism (Yegani and Korver, 2010, Casadevall et al, 2004). We produce the IgY against TNFR1 whole protein which was efficiently recognizedand the acceptable amount of IgY was obtained.

Material and methods

RNA extraction

RNA extraction was done with an RNx kit, following manufacturers' instruction. Approximately 1 mL from isolated white blood cells was dissolved in the equal volume of RNx solution and gently vortexed for 5–10 seconds then kept in room temperature for 5 minutes. 200 µl chloroform was added and mixed for 15 minutes. Then the microtube took on the ice for 5 minute. In order to discard the other part of the cell except RNAs, the solution was centrifuged at 12000 rpm for 15 minutes at 4 °C. After centrifuging at12000 rpm for 5 minutes at 4 °C, we discarded the supernatant and added equal volume of 70 % ethanol. In the other step, the ions were removed by centrifuging at 7500 rpm for 8 minutes. The last step was dissolving in diethylpyrocarbonate water.

cDNA synthesis

CDNA template was constructed according to an Intron kit procedure, by using oligo (dT) and hexamer primers. The mixture of 0.5 µl of oligo (dT), 0.5 µl random hexamer primers, 1000 ng RNA and distilled water was incubated at 65 °C for 5 minutes, then dNTP (2 µl), reverse transcriptase (1 µl), ribolock (1 µl), PCR buffer (4 µl) and water were added. We put the mixture back to termocycler and cDNA synthesis was performed at 65 °C (5 minutes), 25 °C (5 minutes), 42 °C (60 minutes) and 70 °C (5 minutes).

Polymerase chain reaction and cloning

PCR amplification was performed with 50-µl reaction mixtures containing 5 µl of 10× PCR buffer, 2 µl of a deoxynucleotide triphosphate mixture (2.5 mM each dATP, dCTP, dGTP, and dTTP), 1.5 µl of each oligonucleotide primer, 1 µl of pfu DNA polymerase (fermentase), and 0.5 µl of cDNA. The PCR was performed with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 45 s 94 °C, 30s 54 °C, 60 s 72 °C, and end step for 10 min at 72 °C. The final cycle was 10 min at 72 °C and was included to ensure full extension of the product. The PCR product and pET28a plasmid were digested with *Eco*RI enzyme. After ligation reaction the recombinant vector was transferred to competent BL21 cells (with Chloride calcium).

Expression and purification of TNFR1

Colonies with recombinant plasmids were cultured in LB broth media containing 50 µg/ml Kanamycin and then incubated at 37 °C. Thereafter, Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the cultures. In the last step of purification recombinant protein was extracted by Ni-NTA affinity chromatography.

SDS-PAGE analysis of TNFR1 protein

This gel analysis was carried out according to the method of Laemmli. 15 % PAGE was made using Bio-Rad Mini Protein system. 20 µl of IgY-TNFR1 was diluted at 1 : 3 ratio with sample buffer then the mixture was heated for 10 minutes at 100 °C. 20 µl of sample was loaded into each well. Protein molecular marker (Fermentas) was used as a standard molecular weight marker.

Immunization of hens

Immunization of hens was performed in animal house of Pastour institute (Tehran, Iran) with a total of 250 µg protein plus equal volume of Freound adjuvant per animal by intradermal injection in multiple sites of breath, followed by three subsequent boosters.

Isolation and purification of IgY

The PEG isolation protocol was used to purify of the IgY. In the first step, it was carried out by separation of the egg yolk from the white part. Then the mixture was added to two volumes of phosphate buffer. The solution was then mixed with magnetic stirrer. In the other step, two volumes of chloroform were added and were incubated at 4 °C for 20 minutes. Then the solution was centrifuged for 20 minutes at 14,000 g. After centrifugation the supernatant was collected and the pellet was discarded. In the whole molecular weight of the solution 12 % (w/v) PEG6000 was added to the supernatant and mixed with magnetic stirrer for 20 minutes to remove lipoproteins. Again to remove the contamination and receive the best purity the mixture was centrifuged at 14000 ×g, 10 minutes at 4 °C and the supernatant was discarded and then dissolved in PBS. The sediment was then resuspended in equal volume of phosphate buffer and preserved at 4 °C until further use.

Elisa analysis of IgY

Ninety-six-well ELISA plates were coated O/N with 20 µg of either recombinant protein purified and solved in 100 µl of coating buffer and incubated at 4 °C over night for binding. The empty sites were blocked by adding 200 µl per well of 1 % bovine serum albumin in PBS and the Plate was incubated at 37 °C for 1 hour. Plate was subsequently washed with PBST and incubated with 100 µl IgY-extract at appropriate dilutions (dilutions 12 to 0.325 µg/ml were used). Plate was incubated for one hour at 37 °C and subsequently washed with PBST. For the chicken antibodies 100 µl of diluted (1:1000) goat anti-chicken immunoglobulin coupled to horseradish peroxidase was added and the plate was incubated for 1 hour at 37 °C. We have washed it in every step for 3 times with PBST. In the detection step, enzyme activity was determined by adding 100 µl of freshly prepared substrate solution (TMB with sterile H_20_2) and the plate was allowed to stand at room temperature in the dark for 20 minutes. The reaction was

stopped by adding1 M hydrochloric acid and the plate was read at 450nm in an ELISA reader.

Western blot analysis

The protein quality and quantity were measured with western blotting assay. 20 µg/ml from purified protein was loaded in each well and separated with SDS-PAGE gel electrophoresis (15 %). Then TNFR1 protein was transferred to nitrocellulous membrane and incubated with the first antibody (anti-his tag mouse antibody). After finishing the first antibody attachment, the membrane was incubated with secondary antibody (anti- anti IgG HRP conjugated). Bands were visualized using the chromogenic substrate, ECL.

Result

PCR amplification and cloning of TNFR1 gene

Cloning of the desired gene from template was started with polymerase chain reaction. In mammalian cellules according to transcription post modification process, we need to delete introns in vitro and just amplify exons. So, we used mRNA. After extraction of mRNA from human white blood cells, cDNA was synthesized. The prepared cDNA was used as a template to amplify the expected gene (*TNFR1*). As you can see in Figure 1, the desired primers were specially attached the used template and PCR reaction was successfully done. And the *TNFR1* gene with 1402 bp length was produced in the end of reaction. The gel electrophoresis results confirmed it.

Expression and purification of TNFR1 protein

In the research laboratories to purify desirable proteins which do not have a suitable affinity ligand, they had a problem. One useful way to circumvent this obstacle is to genetically fuse the gene encoding the target protein with a gene encoding a tag which has an affinity. So, the chimeric protein with tag allows for specific capture of the fusion protein in this case, we use his tag (histidine repeat) which can purify with Ni-NTA affinity chromatography column. As it is shown in Figure 2, the solution which has an 8 M urea remarkably washed the protein and specially brought out the desired protein, TNFR1. It has almost 47 kDa molecular weight that is demonstrated with the protein molecular weight marker.

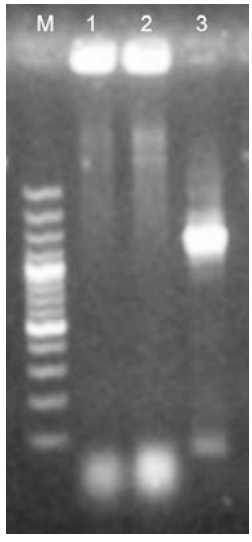

Fig. 1. PCR amplification of human TNFR1 from extracted cDNA. The size of the DNA sample I lane 3 is 1402 bp. Lanes 1 and 2 show negative reaction (without primers and with bacterial genome). Lane M is the DNA molecular weight (100 base pair plus).

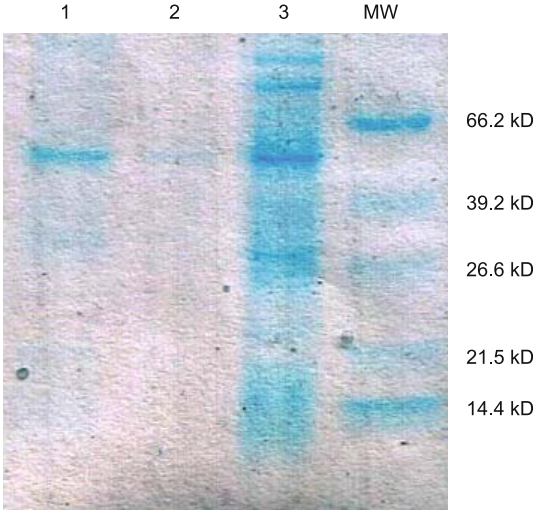

Fig. 2. SDS-PAGE electrophoresis results. A TNFR1 gene was cloned in prokaryotic expression vector, pET28a and DNA was transformed in to *E. coli* BL21(DE3) competent cells. The SDS-PAGE gel shows protein expression purification with Ni-NTA column. In this figure, lane MW is protein molecular weight marker, lane 1, lane 2 and lane 3 are samples. The lane 1 shows almost 100 % purity.

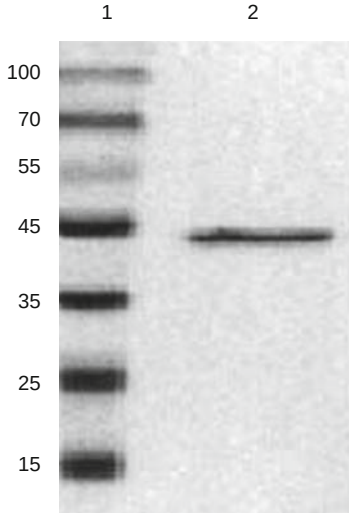

Fig. 3. The western blot analyzing results. The molecular weight marker with 8 bonds is in the first lane from very left and the second lane with 44 kDa protein. The interaction of substrate with ECL colored the membrane.

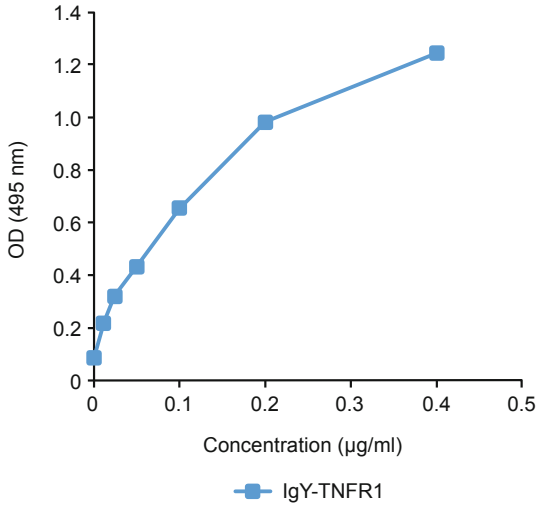

Fig. 4. ELISA result. Antibody responses in hens which is accumulate in egg yolk. After purification of egg yolk antibody their specificity were examined with ELISA assay. As you can see, their affinity to attach the TNFR1 protein increases as the concentration increase.

Western blotting assay and confirmation of TNFR1 protein

Western blot is a technique that is very useful for protein detection as it allows the user to quantify the protein expression as well. Western blot is widely used for detection and analysis of protein, is shown in Figure 3, after separation of protein which was purified with Ni- NTA column with electrophoresis according to polypeptide length (under denaturing condition), the desired protein was transferred to a membrane and TNFR1 protein was attached to mouse antibody against his-tag. The resultant sandwich (antigen-antibody), both, recognized with the secondary HRP conjugated antibody (anti-IgG) and at last confirmed the accuracy of cloned and purified protein, TNFR1. As you can see the bond with almost 44 kDa molecular weight in the membrane was colored with substrate which interacted with the mentioned sandwich.

ELISA results of IgY specificity

ELISA assay was performed to stimulate antibody production and its specificity. Furthermore, after adding the purified IgYs to the coated wells, they were attached to peptides (Fig. 3). As you can see in Figure 4 where Antibodies concentration increase, the attachment of them to the TNFR1 protein also increases. The specificity of antibodies considerably depends on its concentration growth.

Discussion

IgY has many uses, the most basic and the most common applications are in veterinary medicine (Mathew et al, 2007). The interesting thing about the IgY is its use as a food supplement beside its diagnostic and treatment application which is because of its resistance against enzymatic digestion in the body (Cook and Trott, 2010). The IgY generated against a number of pathogenic intestinal bacteria has been used in animals such as bacterial antigen in *E.coli* (Yokoyama et al, 1992, DeLoney and Schiller, 2000), Bovin rotavirus (Kuroki et al, 1993, Vega et al, 2011), Salmonella enteritidis (Rahimi et al, 2007), S. thyphimuruim (Chalghoumi et al, 2009), bovin mastitis (Wang et al, 2011).These types of antibodies are tested on a variety of animals such as pigs, cattle, poultry, dogs and in the humans. The effect of IgY against a number of human pathogens evaluated both in vitro and in clinical studies on laboratory animals was shown and it was very effective (Kovacs-Nolan and Mine, 2012). There are some examples of preventing successful cloning of Pseudomonas aeruginosa in the respiratory tract of patients with cyctic fibrosis (Nilsson et al, 2008, Nilsson and Larsson, 2007), H. pylori on the digestive system (Attallah et al, 2009), Porpbyromonas gingivalis on teeth (Yokoyama et al, 2007), Streptococcus mutans demolition agent in human teeth (Kruger et al, 2004), and also in treatment of *E. coli* infections in humans (Cook et al, 2007) and anti-shiga like toxin I (Wang et al, 2011). In inflammatory bowel disease (IBD), tumor necrosis factor, one of the inflammatory cytokine is involved (Garside, 1999). Due to the high costs and risks of usage of monoclonal antibodies (Sandborn and Hanauer, 1999) the use of IgY against TNFα by Worledge in 2000 could solve these problems (Worledge et al, 2000). According to the expanding world of IgY and its charm, it is a good choice for use in the treatment of cancer.

The researchers often uses western blot to separate and recognizes the desired protein in the research process. However, in the mixture of proteins after separation based on their molecular weight, the produced TNFR1 protein was confirmed. This protein has an acceptable specificity which makes us sure to use it as a correct protein to immunize the hens. As we mentioned before, the whole protein is recognized with anti his-tag antibody which attach to the protein with his-tag. We used this tag to purify our protein from the whole continent of bacterial proteins, so according to this, the protein with just 44 kDa molecular weight if reacting with anti his-tag antibody will be the point antigen.

Interestingly, the produced protein effectively stimulated the immune system of hens. In this study our produced antibody was as same as the one produced in mouse and at the end of the production process, the appropriate antibody was obtained.

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