Cytotoxicity Effects of Mouse IgG Produced against Three Nanoliposomal Human DR5 Receptor Epitopes on Breast Cancer Cells

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Abstract

Cancer causes cells to avoid death while being the second cause of death in the world itself. Damaged cells in the absence of apoptosis will increasingly amplify their inefficient genome. Of the two main apoptosis inducing pathways in cells, the first has p53 protein as the main initiating factor in the cascade. According to research results this protein is mutated in 50% of cancers and sointerest has concentrated on the second pathway that features death receptors. Among these receptors TRAIL1/DR5 is especially expressed in cancer cells. So targeting such receptors can initiate the apoptotic cascade in cells. Interestingly by substitution of activating ligands with antibodies as agonists, we could efficiently turn on the apoptosis pathway. First of all, three small peptides from the DR5 protein extracellular domain were synthesized and injected with two different kind of adjuvants (Fround and liposomal encapsulation) separately into mice at 15 day intervals. As a result, liposomal peptides induced the immune system more efficient than Frounds adjuvant and at the end point the antibodies which were obtained from liposomal peptide injection induced much more effective death. Liposomal formal could be used as an adjuvant in immunization utilizing small peptides. They carry, protect and deliver peptides very efficiently. In addition, small peptides of a certain size from the extracellular domain of DR5 proteins not only can induce immune system but also produce antibodies playing a remarkable anti-cancer roles against breast cancer cells (MCF-7).

Keywords: Cancer – apoptosis - DR5, trail – liposome - adjuvant

Introduction

Cancer is a genetic disease in which genomic mutations cause deregulation of cell critical processes including growth and death. At finally, it leads to the uncontrolled cell growth without death and cancerous cells birth (Bellail et al., 2009). One of the most interested pathways of cancer treatment is apoptosis induction. Apoptosis is the best way of evoking death especially in targeted cells with minimum side effects (Reed, 2006). Apoptosis is a genetically programmed biochemical process that removes unwanted cells and maintains tissue homeostasis under physiological and pathological conditions. practically, there are two pathways that control the initiation of apoptosis: the death receptor-mediated extrinsic pathway and the mitochondria-involved intrinsic pathway (Benedict et al., 2003). TNF receptor superfamily regulates the apoptosis in the first pathway. The extracellular domain of TNF receptors has cystein rich domains (CRDs) which conclude six cystein repeat and three or four disulfide bonds consequently (Bodmer et al., 2002; Cha et al., 2000). Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) or Apo2 ligand (TRAIL/Apo2) is a member of TNF family and discovered by Wiley et al., in 1995, searching TNF family proteins homologouses in EST database (Wiley et al., 1995; Mahalingam et al., 2009). This protein binds to the DR4 (Pan et al., 1997; Schneider et al., 1997 ) and DR5 (Chaudhary et al., 1997; Walczak et al., 1997; Wu et al., 1997) receptors and activates apoptotic pathways selectively in cancerous cells (Ashkenazi, 2002). Specific killing activity of TRAIL on various tumor cells has brought about great interests to this target or its antagonists in clinical applications (Wei et al., 2005 ). DR5 was described more contributing than DR4 in the overall apoptotic activity of TRAIL during apoptosis (Kelley et al., 2005; Almasan and Ashkenazi, 2003; Ichikawa et al., 2001; Peto et al., 2000). One of the best antagonists of this protein is

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specific antibodies produced against the receptors which can attach to its antigen, the death receptor, and initiate the intrinsic pathway of apoptosis. In order to produce antibody, mammalian animals are susceptible candidates. The whole aim of this project was to induce apoptosis in cancerous cells by the aim of antibodies which were produced against death receptors. According to this, firstly three small parts of extracellular domain of DR5 receptor were produced and then injected to mice. Finally, the antibody elevation were evaluated in body.

Material and Methods

Peptide synthesis: Three small part of extracellular domain of the DR5 protein that contains 15 (CDSGE VelSpCTTTTR), 21 (SCKYQD YSTHWN DLLF CLRC) and 27 (NTVCQCEEGTFFREDSPMCRCRTGC) amino acids are produced in peptide synthesis center of National Institute of Genetic Engineering & Biotechnology, Iran.

Liposome production: In order to produce liposome, egg phosphatidylcholine and cholesterol (10:1 w/w) were dissolved in 50 ml ethanol. The mixture shaked on stirrer for 5 hours, until homogenized suspension obtained. Then ethanol content was evaporated and the gelose that attach to the round-bottom was resolved in 20 ml sterile PBS. The resulting suspension was sonicated in 600 hearts for 5 minutes, then there was added 5 mg dextran and the resulted mixture allocated in 2 ml microtubes, and were restored in -200C. The efficiency and sizing of the produced liposomes were analysed by Zetasizer Nano.

Immunization: Immunization of mice (Balb-C, 10 for each group) was performed in two groups for each peptide, in Pastour institute (Tehran, Iran) animal house. First group was immunized with 350 μg peptide combined with Freund’s adjuvant and second group were injected liposomes that contain total 350 µg of each peptide per animal by intradermal and intrarectal injection in multiple sites, followed by three subsequent boosters.

Serum collection: In this section blood were collected and incubated in 370C for 1 hour and then they were kept in 40C overnight. After 24 hours, they were collected by centrifugation at 1000 rpm 3min. The samples were stored in -200C until they were applied for ELISA assay.

ELISA assay: 20 μg of DR5 protein (produced in Pastour, Pilot labrotoary) as an antigen were stuck onto a plastic surface of 96-well plates and incubated overnight at 40C. After removing the supernatant, 200 μl of BSA which was dissolved in PBST (PBS+ 05% Tween-20) as a blocking buffer was added and after 1 hour incubation in 370C, the solution were discard. The serial dilution (dilutions 1:1,000 to 1:64,000) of obtained antibodies (100 μl per well) were added and incubated for 30 minutes. Supernatant were discarded and incubated the sandwicjh with secondary antibody (anti-IgG) HRP-conjugated (100 μl). After 30 minutes and removing the solution, the reaction was detected by the marker changing colour TMB buffer and the reaction was stoped with 1% HCL. The absorbance was read in 450 nm with ELISA reader. In each step the wells were washed with 200 μl PBST three times.

MTT Assay: The MTT method of cell determination was done in three days. First 10,000 MCF7 cells (human breast cancer cells) were cultured per well in the first day and they were incubated for 24 hours. In the second day, the media were removed carefully and the cells were treated by antibodies (final volume should be 200 μl). In the last day, the supernatant were removed and then added 100 μl of 5 mg/ml MTT solution to each well. The cells were incubated for 2 hours at 370C, 5% CO2 in culture incubator. The media were carfully removed and the color were resolved in MTT solvent (isopropanol). The absorbance of the color were read at 590 nm. Then, the provided data were calculated with Pharms software.

Statistics: All data is expressed as mean ± S.D. Differences between experimental and control groups were determined by using the T- test. Values of P < 0.05 were considered significant.

Results

The liposome produced was in the nano scale range. Liposomes size were measured with Zetasizer Nano at 25°C (Figure 1). Sizing measurements were made on the neat liposome samples, where the samples were diluted with PBS almost 0.5 nm (OD). Liposomes without any cargo had 73.55 nm diameter but after loading peptide in each liposom production reaction, it grows till 360.9 nm. Therefore, such differentiation in size scales demonstrates the loading of peptide in liposomes.

Obtained antibodies from two kind of injection were definitely recognize their antigens.

After the immunization of mice and blood sampling, the serum were separated. ELISA results demonstrated that not only antibody was produced but also they can

Figure 1. A Histogram of Liposom Size Distribution Determined by DLS. The Diameter Shown is the Hydrodynamic Diameter of the Free Liposome (A) and Liposomes Loaded With Peptides (B)
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Discussion

The induction of death in the cell without control, like cancerous cells can be helpful in deleting them. Among two main death pathways (necrosis and apoptosis) in mammalian cells, it seems apoptosis is the main pathway. Specially the dead receptors which are induced through dead receptors could be a good candidate.

Over the last thirty years, breast cancer treatment has gradually obtain many successful event, such as early detection effectiveness and improvements. In contrast, researches have led to a significant decrease in breast cancer therapy in loco-reginals and systemic therapy (Gajewski, 2007) Therefore, in order to increase the effectiveness of systemic treatment, targeted therapy have been exclusively investigated over the past two decades. Nowadays, it is demonstrated that one of the most promising therapeutic agents and targetable molecules are dead receptors and their ligand and agonists of course. Activating the TRAIL pathway with an alternative approach and the use of specific agonist antibodies (Ichikawa et al., 2001) is interesting. In such kinds of activation, they use the ligand receptor specificity and after attachment, biomimic and prolonged real ligand mechanism inducing in cells takes place. Monoclonal antibodies, as one of such agonists, like, rituximab, terastuzamab and beracizumabare now widely applied in clinic and research based works for cancer therapy (Mori et al., 2004). Beyond DR4, DR5 is the predominant TRAIL receptor, which is expressed at the cell surface and mediates apoptotic signals in human’s cancerous cells (Amirijavid and Hashemi, 2015 and Amirijavid and Hashemi, 2014).

However, IgG obtained from immunized mice against DR5 as it demonstrated, could specially induce dead.
According to the peptide small size, we supposed that the diversity of the varied domains of antibody were restricted. It means although the scale of IgGs was obtained (polyclonal antibody) their variety to identify the antigens limitation. So, it can help to reduce the unspecific answers in body. Using two kind of adjuvants help us to obtain more antibody. According the small size of injected peptides, the kind of induction in mice immune response was important. Fraud’s adjuvant as a convenient and useful adjuvant for immunization with protein, was the first choice. Though, liposomal peptides was interesting too. Liposome as a carrier was expected to protect active compounds against chemical and enzymatic attacks in the body. In other word, they were easily produced in large scale and provide longer drug half lives as well as tailored drug-release profile, reducing high peak plasma concentrations (Amirijavid and Hashemi, 2015; Amirijavid and Hashemi, 2014; Allon et al., 2012). Moreover, liposomes which loaded with our peptides, play an important role. They carry large amount of peptide safely to the body and deliver their cargo to the cytoplasm through conjugation with cellular membrane. Indeed, they not only do transport the peptide protectively in intracellular spaces, but also deliver large amount of simultaneously in the intracellular space. Furthermore, it can increase cell uptake and subsequently, influences antibody production growth. The results emphasize the efficiency of using such kind of immunization in contrast to using Fraud’s adjuvant.

In addition, in the same concentration of antibody (each from two kinds), we have different interaction manners. Antibody produced against the 15 aminocacids peptide in a liposomal form (15L) had the best answer among all antibodies. This is in contrast to its counterpart in injection with Fraud’s adjuvant (15A). According to the petide scale, by decreasing the peptides size, the possibility of uptake and introduce of peptides to cell surface and at least producing antibody was declined by APC cells obviously. But, liposome protects peptide against degredation and increase delivery of enough amount to APC cells. On the other hand, the small size of the antigen can specify the antibody recognition site and this lead to increase in the subsequent response. However, in 21 aminocacids peptides in liposomal compound (21L) the same correlation can be seen also. Therefore, cells uptake peptides easier with more possibility, by growing peptide size. So, in the first kind of injection, not only does mouse have the most amount of antibody production, but also the produced antibodies obtain the first killing step.

As a final conclusion, it is interesting to note that liposomal formol could be used as an adjuvant in immunization with small peptides. They carry, protect and deliver peptides as efficient as possible. In addition, the small peptides not only can induce immune system but also the produced antibodies can play a remarkable anti-cancer role.

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