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Research Article

Production and Verification of Anti-Tumor Activity of Monoclonal Anti-EGFR-Recombinant PE38 Immunotoxin in A431 Tumor Cells

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Abstract

Background: Tumor growth and progression depend largely on the activity of cell membrane receptors like epidermal growth factor receptor (EGFR). This receptor plays a significant role in the growth and survival of many solid tumors. Its biological feature makes it a highly appealing target for cancer treatment. On the other hand, immunotherapy is an efficient approach in cancer treatment, and immunotoxins have a predominant position herein. Thus, this approach can be used in high EGFR-expressed cancer therapy.

Methods: In this study, the production of monoclonal anti-EGFR-recombinant PE38 was used as the special treatment against EGFR-activated cancers. For this purpose, the A431 cell line originating from a squamous carcinoma was used. In order the production of this immunotoxin, the toxin was conjugated with an antibody by chemical method. To confirm conjugation and its purity, SDS-PAGE was performed by immunotoxin electrophoresis. Then, the antitumor effects of immunotoxin in the induction of apoptosis of tumoral cells were assessed by the ELISA method.

Results: The conjugation and purity of immunotoxin were confirmed by immunotoxin electrophoresis. Also, the ELISA results indicate that the produced immunotoxin induced 62% antigen-specific apoptosis ($P<0.0001$) in tumoral cells compared to the control cells.

Conclusion: To conclude, our study provides a promising therapeutic approach against EGFR-associated cancers and our individual immunotoxin can be used in the treatment of tumors with membranous EGFR.

Introduction

The growth and spread of tumors, like normal cells, are highly dependent on the activity of cell membrane receptors that control intracellular signal transduction pathways. These receptors control many different physiological processes of the cells. As One of the most important types of these cell membrane receptors, the epidermal growth factor receptor (EGFR) plays a pivotal role in the growth and survival of many different types of tumors.[1,2] Activation of EGFR triggers many cellular events that eventually lead to cell proliferation, differentiation, migration, adhesion, protection against apoptosis, and induction of angiogenesis, so inhibition of EGFR can inhibit cancer-related properties.[3,4]

Monoclonal antibodies have been widely used in clinical trials to deliver cytotoxic agents to tumor cells. Antitumor effects of Cetuximab monoclonal antibody against EGFR have been reported in many carcinomas such as colorectal, prostate, breast, renal, pancreatic, head and neck carcinoma, and non-small cell lung carcinoma.[5–7] The antibody was approved by the US Food and Drug Administration (FDA) for the treatment of advanced metastatic colorectal cancer in 2004.[8–10] In vivo tests on A431 cell xenografts in an animal model showed that the antitumor effects of concomitant treatment of EGFR monoclonal antibodies with Doxorubicin produce more effective results together than when used alone and lead to tumor removal in all animals, while treatment of each of these alone only reduces tumor growth.[11]

One of the promising approaches in cancer therapy is the production of immunotoxins as cytotoxic agents targeting cell surface molecules that enter the cell and subsequently lead to cell death.[12,13] Immunotoxins consist of a targeting unit such as a ligand or antibody that is specific to a particular cell type and a toxin to which it is attached. The target unit identifies cell surface molecules and delivers the whole molecule to specific cancer cell surface receptors. The toxin then induces cell death by

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reaching the cytosol and inactivating living cell processes or by altering the tumor cell membrane integrity.[14] PE is a toxin secreted by Pseudomonas that inhibits protein synthesis and causes cell death through apoptosis.[15–18] The immunotoxin RFB4 (dsFV) -PE38 (BL22) targets CD22, which is expressed in B-cell lymphomas and leukemias. In this immunotoxin, the anti-CD22 antibody binds to PE38 and is used to treat B cell malignancies such as HCL, CLL, and some B cell lymphomas. This immunotoxin elicits a 73% therapeutic response in cancer patients.[14,19,20] Our immunotoxin of interest is composed of Cetuximab and PE38. This study aimed to produce and evaluate anti-EGFR immunotoxin and evaluate its effects in the induction of apoptosis in cancer cells.

Methods

Cell culture

The A431 cells were purchased from Pastor Institute in Iran (Iran, Tehran) and cultured in RPMI-1640 medium, supplemented with 10% FBS (Gibco). The medium was later added 1% antibiotic (penicillin 100 IU/ml, streptomycin 100 µg/ml) and sterilized by 0.22 µm filters. The cells were kept in the standard incubator at 37 °C. The media was changed every 2 days until the cells entered the logarithm phase. Then they were ready to be used for the tests according to our previous study.[21]

Production of monoclonal Anti-EGFR-recombinant PE38

For the conjugation of anti-EGFR antibody[22] with recombinant PE38 (rPE38) toxin,[23] the formation of disulfide or thioether bonds between the two molecules was used. According to this protocol, sulfhydryl groups in antibodies were generated using 2-Iminothiolane and then attached to the toxin via a thioether bond.[24] First, the PBS monoclonal antibody buffer was replaced with 0.2 M sodium phosphate buffer (pH7.0) containing 1mM EDTA by dialysis bag. PBS toxin PE38 buffer was then replaced with 0.2M sodium phosphate buffer (pH 8.0) containing 1mM EDTA by dialysis bag. The three-fold monoclonal antibody was mixed with 2-Iminothiolane, HCl (SIGMA, I6256, Germany) and incubated in C37 for one hour. PE38 was also mixed with SMCC (SIGMA, M5525, Germany) three times and incubated for 30 minutes at room temperature. Finally, they were removed from the antibody and PE38 by Iminothiolane dialysis bag and free SMCC. PE38 was then added to the antibody in a ratio of 4 to 1 and incubated for 20 hours at room temperature to be ligated by theatrical grafts. Immunotoxins were isolated from free antibodies and free PEs by gel filtration (Sephadex-G-200). Finally, the immunotoxin was sterilized by filtration through 0.2 µ filters.

Confirmation of conjugation by SDS-PAGE

SDS-PAGE and Kumasi Blue staining were used to confirm conjugation of anti-EGFR antibody with PE38. SDS-PAGE experiment performed based on the previously reported method by Green and Sambrook.[25] 10% resolving gel was prepared using recommended volumes of SDS-PAGE components, which were electrophoretically reduced. After polymerization, the gel was washed several times with distilled water. The stacking gel was prepared and poured it over the polymerized resolving gel. The samples were then mixed with sample buffer containing β-mercaptoethanol and heated at 95 °C for 10 min for reducing and for non-reducing samples are mixed with sample buffer alone preceding to load on to the gel. Finally, a total volume of 10 µl of samples was mixed 2 µl sample buffer to load onto the gel along with a prestained protein marker (Figure 1).

Evaluation of apoptosis by ELISA method

Roche cell death detection ELISA kit (Cat.no.11 774 425 001) was used to measure the amount of apoptosis and necrosis caused by immunotoxin. The basis of this approach is the measurement of histone-DNA complex fragments in cell lysis and supernatant of cell culture and was performed according to the kit protocol. First, A431 cells were cultured in 3000 cells per well from 96-well plates for 24 hours. Then a certain concentration of immunotoxin was added to each well. Cells were incubated for 5 days in a standard incubator with 5% CO2. Later, the cells were collected from the wells to measure the amount of necrosis and apoptosis. Supernatant and cell lysis were added to streptavidin-coated microplates. Immuno-reagents were then added to each well from the microplate and incubated for 2 hours at room temperature. After adding the substrate and then stopping the reaction, the adsorption of the wells at 405 nm was measured by the ELISA reader. We have used PBS as negative control and camptothecin as a positive control.

Statistical analysis

All data are shown as the mean ± SEM. GraphPad Prism 6 software (San Diego, CA, USA) was used for statistical analysis. Student's t-test and one-way analysis of variance were done to demonstrate statistical differences among groups, followed by the Tukey test. The *P* values smaller than 0.05 were considered statistically significant.

Results

Conjugation of anti-EGFR antibody with PE38

After activation of antibodies with 2-Iminothiolane and toxin with SMCC, these two proteins were conjugated together. To confirm conjugation and its purity, SDS-PAGE was performed by immunotoxin electrophoresis. The results demonstrated that the immunotoxin band that appears at 190 kDa indicates successful and pure conjugation (Figure 1).

Evaluation of induction of apoptosis and necrosis by immunotoxin by ELISA method

ELISA method was used to measure the induction of apoptosis and necrosis by immunotoxin. In this

experiment, A431 cells with high EGFR expression were exposed to immunotoxin for 5 days. After that, the results showed that a 5-day incubation time and concentration of 2 µg/100µl immunotoxins had the greatest effect on inducing apoptosis in the cell, and immunotoxin at these concentrations did not cause necrosis (Figure 2). In this experiment, the concentration of 2 µg/100µl had the greatest effect on inducing cell apoptosis and reducing cell growth ($P<0.0001$). The immunotoxin caused 62% apoptosis in A431 cells compared to the control.

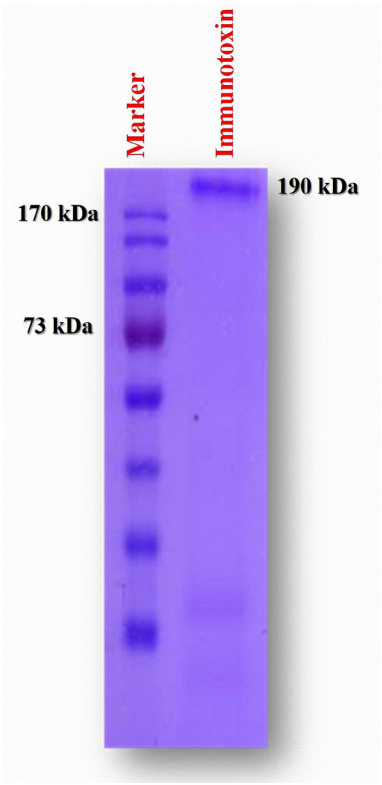

Figure 1. The SDS-PAGE method, which is an analytical technique to separate proteins based on their molecular weight, was successfully applied to investigate the existence of immunotoxin. The immunotoxin band appears at 190 kDa in all of the prepared samples

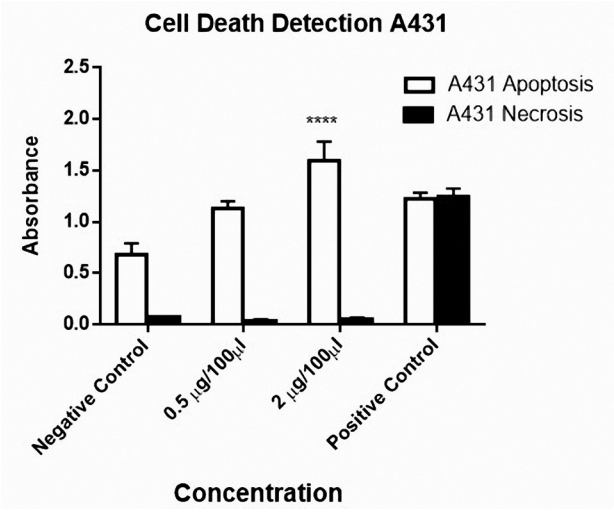

Figure 2. Evaluation of apoptosis and necrosis of A431 cells. The highest rate of apoptosis was observed in the concentration of 2 µg/100µl of immunotoxin. PBS was used as negative control and camptothecin as a positive control. **** $P<0.0001$

Discussion

Inhibition of EGFR causes many cellular effects and causes antitumor responses so that there is a lot of evidence that EGFR inhibitors are useful for cancer therapy.[22,26,27] The study conducted by Sabrina Oliveira et al. in 2006 showed that blocking of EGFR signaling pathways alone could not inhibit cell proliferation. The cancer cells may use multiple intracellular pathways to overcome the blockage of specific receptors. Therefore, inhibition of alternative pathways and a combination of EGFR inhibitors and other targeting drugs, overall, seem to show better results.[28] Lack of specific effect and limited efficiency of common cytotoxic agents has led to careful design and development of targeted therapies to distinguish between malignant and non-malignant cells. To develop such factors, it is necessary to identify abnormal molecular and biochemical pathways to distinguish malignant cells from non-malignant ones.[29] Immunotoxins are hybrids of proteins that owe specificity to antibodies and cytotoxicity of toxins.[30]

In this study, the cytotoxicity of anti-EGFR immunotoxin was shown against A431 cells, which express high levels of EGFR at their surface. Expression of EGFR in human tumors is associated with a poor prognosis and rapidly progresses to cancer. Therefore, EGFR has been considered a suitable agent for tumor treatment. In immunotoxin-based approaches, to reach the appropriate immunotoxin effect on target cells, the immunotoxin must penetrate the cytoplasm of the cell. In a 2005 study of EGFR-supplying cells in lung cancer, David Raben et al. showed that immunotoxin effects depend on the number of EGFR on the cell surface.[31] Another study by Kazuto Nishio et al. in 2007 examined the effect of Cetuximab on tumor cells and antibody-dependent cellular cytotoxicity (ADCC) activity against several tumor cell lines. the results showed that ADCC-dependent antitumor activity results from the degree of affinity of Cetuximab for the extracellular domain of EGFR and the effects depend on EGFR expression on the tumor cell surface.[32] Besides, a 2009 study by Yong-Feng Yu et al. found that the inhibitory effect of Cetuximab on tumor cells was dependent on the number of EGFR receptors and the concentration of monoclonal antibodies.[33]

Finally, a cell death detection kit was used to determine the type of cell death. This test differentiates apoptosis from necrosis and determines the type of cell death. According to the results of this experiment, the immunotoxin induced apoptosis in A431 cells at the concentrations used and no necrosis occurred in the cells and the recombinant toxin alone had no significant effect on cell growth. The severity of apoptosis is related to the number of cell surface receptors so that in A431 cells, which have a large number of receptors on the surface, immunotoxin induced 62%

of apoptosis compared to the control and is $P<0.0001$. similar to our study, in a 2008 study on melanoma cells, Michael Schwenkert and colleagues reported that the effect of recombinant immunotoxin in inducing apoptosis on A2058 and A375M cells was up to 80%.[34]

Conclusion

Despite new treatment approaches, the clinical outcomes in a substantial number of cancer patients have yet to be improved. According to the results of mAb tests, this antibody can be used in cancer diagnosis and research such as immunoblotting, ELISA kit design, immunofluorescence staining, etc., and also by producing chimeric antibodies. Hybridoma has been used to treat cancer. The results related to immunotoxin also showed that this immunotoxin can be used in the treatment of EGFR-supplying cancers if it is produced recombinantly. Taken together, our results revealed that monoclonal anti-EGFR-rPE38 was effective in suppressing the growth of A431 cells. For the first time, we indicated that this anti-EGFR-rPE38 exerts antitumor activity on tumor cells by inducing apoptosis. Given these, monoclonal anti-EGFR-rPE38 has a great potential to be a promising therapeutic candidate for treating EGFR-associated cancers.

Conflict of Interest

The authors have no conflicts of interest to declare.

Ethical Issues

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research.

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