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Original Article

Construction And Characterization Of Recombinant Adenovirus Expressing Single Chain Antibody Against DEC205 Receptor Fused To Carcino Embryonic Antigen For Preliminary Application In Immunotherapy Of Cancer In Mice

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Abstract

Background and Aims: Selecting appropriate antigens, suitable delivery vehicles and proper immune adjuvants are important items in vaccine designing. According to the previous studies, Carcinoembryonic antigen (CEA) and Adenoviral vector are considered as proper antigen and reliable delivery vehicle for cancer vaccines, respectively. Directing antigens to DCs with antibody single-chain fragment variable (scFv) against to DEC-205 (scDEC) can play the role of adjuvant. The aim of this study was to produce a construct encoding CEA targeted to DCs by scDEC in Adenovirus vector platform.

Materials and Methods: scDEC were cloned into p-track-CEA-GFP. Linearized p-track-scDEC-CEA-GFP and pAdenoVator ΔE1/E3 were co-transformed into BJ5183 cells. Resulting pAd scDEC-CEA-GFP was transfected into HEK293 cells to produce Adenoviruses (Ad scDEC-CEA-GFP). The presence of scDEC-CEA gene in the recombinant virus and expression of CEA protein was detected by PCR and electrochemiluminescence tests, respectively. Flow cytometry tests were used to confirm the attachment of scDEC-CEA protein to DCs. Adenovirus infectivity in cells other than HEK293 was confirmed by transduction of Ad scDEC-CEA-GFP into L929 cells.

Results: PCR test showed the presence of scDEC-CEA gene in adenovirus genome. Electrochemiluminescence and flow cytometry analysis confirmed expression of CEA protein and attachment of scDEC-CEA protein to DCs, respectively. GFP expression in L929 cells indicated Ad scDEC-CEA-GFP could effectively infect cells other than HEK293.

Conclusion: As construction of a suitable construct for immunotherapy is a major goal of many research groups, the Ad scDEC-CEA-GFP engineered in this study, which properly targets DCs, can be applied in future in vivo cancer immunotherapy investigations in mice.

Keywords: Cancer immunotherapy, CEA, DEC205, Adenoviral vector

Introduction

Cancer immunotherapy is a potential and promising order in combination with standard treatments like chemotherapy, radiotherapy and surgery (1, 2). Various immunotherapy methods have been studied in this field, one of which is cancer vaccines (1, 3). Several therapeutic vaccine approaches including autologous vaccines, peptide vaccines, viral vector vaccines and dendritic cell (DC) vaccines have been used in multiple tumor types (3). Selecting appropriate antigens, suitable delivery vehicles and proper immune adjuvants are three important elements in designing an effective vaccine (4). Tumor-associated antigens (TAA) are overe-xpressed antigens in cancer cells (4). Some studies about TAA vaccines showed increased immune responses against tumors (3, 5).

Carcinoembryonic antigen (CEA), an over-expressed TAA in colorectal, lung, breast and

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pancreas cancers (6), is considered as a target for cancer vaccines.

CEA targeting studies through therapeutic vaccination approaches have been shown induction of suitable humoral and cellular immune responses (7).

Adenoviral vectors are one of the reliable delivery vehicles as they are safe and rare pathogen to human, which can infect wide range of dividing and resting cells, their genome can accept large cDNAs, the large-scale production is easy and they can elicit an effective antibody and T cell responses against foreign inserted gene (5, 8). So Adenoviral vector could be a proper choice for carrying the CEA gene.

A strategy to improve immune responses against TAAs could be targeting them into dendritic cells (DCs). DCs are antigen presenting cells that provide an essential bridge between innate and adaptive immune responses (9) so amplifying the tropism of antigen to DCs can show adjuvant effect and cause production of more specific and potent immune responses (4). One of endocytosis-mediating receptors on the surface of DCs is DEC-205 (CD205). Studies in which the antigen is directed to DCs with antibody single-chain fragment variable (scFv) against to DEC-205 (scDEC) have shown the improvement of cellular and humoral immune responses (10-14).

The aim of this study was to produce a construct encoding CEA as an appropriate antigen targeted to DCs by scFv against to DEC-205 in Adenovirus vector platform, which can be used for cancer immunotherapy.

Materials and Methods

Primers, Plasmids, Bacteria, Cells, Enzymes, Kits

All primers were designed using Gene Runner software (Version 6.5.47 x64 Beta) and synthesized by Pishgam Co. (Tehran, Iran).

pV-scDEC-pOLLAhis that contain the sequences of antibody single-chain fragment variable (scFv) against DEC-205 (p-scDEC), was a gift from Professor Mathias Tenbusch (Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany) (15). pCMV-CEA-Linker-GFP sparkTag (p-CEA-GFP) was synthesized by Sino Biological company. Low-passage Human Embryonic Kidney 293 (HEK293) cells and L929 cells were obtained from the Iranian Biological Resource Center. The enzymes were purchased as followed: AccuPOL DNA polymerase (AMPLIQON, Denmark), 2X Taq PCR Pre-Mix 2 (BIOFACT, Korea), KpnI restriction enzyme (TaKaRa, USA), Alkaline Phosphatase, PmeI and PacI restriction enzyme (Thermo Scieific, US), T4 DNA ligase (SinaClone, Iran). DNA gel extraction kit and DNA plasmid extraction kit were purchased from Gene All, Seoul, KOREA. DNA Ladder Marker was purchased from SMOBio, Tiawan. The CEA protein expression was analyzed by electrochemiluminescence (ECL) (Elecsys CEA Kit) test. The cell culture related products (Dulbecco, s modified Eagle, s medium (DMEM), FBS, PEN-STREP (100X) and Trypsin-EDTA (1X)) were prepared from BIO-IDEA company, Iran.

scDEC PCR Amplification

PCR amplification of scDEC+G4S Linker was performed using specific primers that were designed based on the sequence of p-scDEC as template. KpnI site was introduced into the sense and antisense primers as underlined: 5'-ggggtaccGCCACCATGGGCTGGTC- 3' (sense), 5'-ggggtaccGCTGTTCCGCACGGCTC-3'(antisense). PCR was performed in a total volume of 50 µl consisting of 1.5 µl DMSO, 1 µl of each forward and reverse primers (10 µM), 0.8 µl dNTPs (2.5 mM), 5 µl 10×polymerase reaction buffer, 2.5U AccuPOL DNA polymerase and 2 µl of p-scDEC (1:100). PCR amplification conditions were as follows: The samples were heated to 95°C for 5 min followed by 95°C for 45 s; 60°C for 25 s; 72°C for 1.5 min, 35 cycles; and 72°C for 5 min as a final extension step.

Subcloning of scDEC into Ad Track Vector

Ad track CMV-CEA-Linker-GFP sparkTag vector (p-track-CEA-GFP) was constructed by subcloning of Carcinoembryonic antigen (CEA) fused to GFP Tag (CEA-GFP) from p-CEA-GFP into pAd track CMV (p-track) in previous works. Both gene and vector were digested with KpnI and ligated after dephosphorylating of the digested plasmid.

The colonies with correct direction of ligation were chosen by colony PCR with primers identifying the end and the first sequences of scDEC and CEA respectively: 5'- ATCGCCA-CCTACTATTGCCAG - 3' (sense), 5'- CA-GGGGATGCACCATCTGTG -3'(antisense). The reaction conditions were as follows: 0.5 µl of each forward and reverse primers (10 µM), 10 µl of 2X Taq PCR Pre-Mix 2 and dH2O to a final volume of 20 µl; 95°C for 6 min; 35 cycles of 95°C for 15s; 62°C for 15 s; 72°C for 30s and 72°C for 5 min as a final extension step. Accuracy of "p-track-scDEC-CEA-GFP" construct was confirmed by scDEC PCR amplification, KpnI restriction map and sequencing.

Construction and Identification of Adenoviral Plasmid

The p-track-scDEC-CEA-GFP was digested and dephosphorylated with PmeI and Alkaline Phosphatase respectively. Linearized purified vector and pAdenoVator ΔE1/E3 DNA were co-transformed into BJ5183 competent cells, that support recombination, by electroporation (eppendorf). The complete reaction volume was plated onto LB/Amp (50 µg/ml) plates. Plasmid DNAs were extracted from ampicillin-resistant single colonies and then were digested with PacI restriction enzyme to select the accurate recombinant Adenoviral plasmid.

Packaging, Production, Amplification and Characterization of Recombinant Adenoviral Vector

Large quantities of recombinant Adenoviral plasmid harboring scDEC-CEA-GFP genes (pAd scDEC-CEA-GFP) were digested with PacI to expose the vectors ITRs (Inverted Terminal Repeats). Purified linearized vectors were transfected into low-passage HEK293 cells, that were cultured in DMEM plus 5% FBS, using Polyethylenimine (PEI) transfection reagent. The cultures were daily observed for CPE and GFP fluorescence. When more than 80% CPE and GFP positive cells were shown (9 days after transfection), the supernatants were collected after three freeze-thaw consecutive cycles. HEK293 cells were infected with collected supernatants to produce more Adenoviruses harboring scDEC-CEA genes (Ad scDEC-CEA-GFP). High volume of virus was collected after centrifuging at 100000 x g for 90 min at 4°C (BECKMAN Model L5-50 Ultracentrifuge). The titer of virus was determined by CCID50 test. In brief, the virus was diluted at a dilution range from 10-2 to 10-10. HEK293 cells that were seeded into a 96-well plate, were infected with diluted viruses. The infected cells were checked in terms of CPE appearance and GFP expression and the titer was calculated by karber formula. All CPE positive wells were confirmed in term of GFP expression.

The presence of scDEC-CEA gene was detected by PCR amplification test with primers that identifying the end and the first sequences of scDEC and CEA respectively. L929 cells were infected with Ad scDEC-CEA-GFP and GFP expression was evaluated under fluorescence microscopy to determine Adenovirus infectivity.

Expression of CEA Proteins by Recombinant Adenoviruses

After 72 hours, the expression of CEA protein was examined by electrochemiluminescence (ECL) (Elecsys CEA Kit) test in supernatants collected from three treatments: infected HEK-293 with Ad scDEC-CEA-GFP, transfected HEK293 with p-CEA-GFP as positive control and noninfected HEK293 cells as negative control.

Evaluation of the scDEC-CEA-GFP Protein Attachment to DCs

HEK293 cells were infected with Ad scDEC-CEA-GFP and recombinant Adenoviruses harboring only CEA-GFP genes (Ad CEA-GFP), separately.

The titer of both viruses were 1×103 CCID50/ml. After 72 hours the supernatants of infected HEK293 cells and untreated cells were collected before and after three freeze-thaw consecutive cycles. The collected supernatants were used for assessing the attachment of scDEC-CEA proteins to DCs. For this purpose, bone marrow (BM)-derived dendritic cells (DCs) were generated as we described previously (16). One milliliter of each supernatant was added to culture of 3×106 immature DCs in 24 wells cell culture plate separately and incubated overnight at 4°C. The attachment of scDEC-CEA to DCs was determined

using fluorescence microscopy and through flow cytometric analysis using the GFP expression levels, as all recombinant Adenoviruses were expressing it.

Results

scDEC PCR Amplification and Subcloning into Ad Track Vector

The amplification of scDEC+G4S Linker was performed by PCR and purified PCR product (885 bp) was cloned into p-track-CEA-GFP.To confirm the successful p-track-scDEC-CEA-GFP construction, the expected PCR amplicon size (885 bp) was confirmed by PCR (Fig I). Besides, following digestion with KpnI, the cloned scDEC segment (885 bp) exited from p-track-scDEC-CEA-GFP (Fig I). The inserted scDEC DNA fragments were also sequenced and analyzed. The result was con-sistent with the p-scDEC sequences (Data not shown).

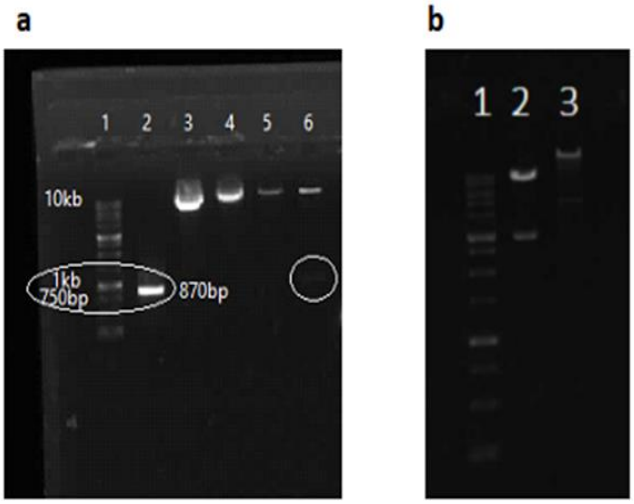

Fig I: Agarose gel electrophoresis figures. (a) cloning of scDEC+G4S Linker PCR product into p-track-CEA-GFP. lane1: 1 KB DNA Ladder Marker, lane 2: scDEC+G4S Linker PCR product with 885 bp size, lane 3: p-track-CEA-GFP, lane 4: p-track-scDEC-CEA-GFP vector that is standing higher than lane 3, lane 5: KpnI digested p-track-CEA-GFP vector, lane 6: KpnI digested p-track-scDEC-CEA-GFP vector that 885 bp DNA fragment was existed. (b) The banding pattern of PacI digested pAdenoVator ΔE1/E3 DNAs. lane1: 1 KB DNA Ladder Marker, lane 2: PacI digested pAdeno-Vator ΔE1/E3 DNA, lane 3: PacI digested pAd-scDEC-CEA-GFP. The banding pattern of DNAs showed larger DNA fragments in PacI digested pAd-scDEC-CEA-GFP that indicated it harboring the scDEC-CEA genes.

Construction and Identification of Adeno-viral Plasmid

pAdenoVator ΔE1/E3 DNA and PmeI digested p-track-scDEC-CEA-GFP vector underwent homologous recombination. To confirm successful recombination, the pAdenoVator ΔE1/E3 DNA and recombinant Adenoviral plasmid were digested with PacI. The banding pattern of DNAs that subjected to agarose gel electrophoresis showed larger DNA fragments in PacI digested recombinant Adenoviral plasmid (pAdeno scDEC-CEA-GFP) that indicated it harboring scDEC-CEA genes (Fig I).

Packaging, Production, Amplification and Characterization of Recombinant Adeno-viral Vector

Linearized pAdeno scDEC-CEA-GFP were transfected into HEK293 cells. When more than 80% CPE and GFP positive cells were shown (Fig II), the freeze-thawed supernatants were collected. The HEK293 cells were re-infected with collected supernatants to produce the Ad scDEC-CEA-GFP. PCR amplification test results confirmed the presence of scDEC-CEA gene in DNA extracted from the infected HEK293 cells.

The CCID50 test was performed on virus collected after high-speed centrifugation and the titer of virus was determined about 1×107 CCID50/ml. Adenovirus infectivity was evaluated by transduction of Ad scDEC-CEA-GFP into L929 cells and GFP expression was seen after 48 hours (Fig II) that indicating recombinant Adenoviruses harboring scDEC-CEA-GFP (Ad scDEC-CEA-GFP) could effectively infect cells other than HEK293.

Expression of CEA Proteins by Recombinant Adenoviruses

The expression of CEA protein was analyzed by electrochemiluminescence test and the results confirmed the presence of CEA in supernatants of infected HEK293 cells (73.2 ng/ml) in comparison with positive control (supernatants of transfected HEK293 with p-CEA-GFP) (376 ng/ml) and negative control (uninfected HEK293 cells) (<0.02 ng/ml).

Evaluation of the scDEC-CEA-GFP Protein Attachment to DCs

The supernatants of HEK293 cells (untreated

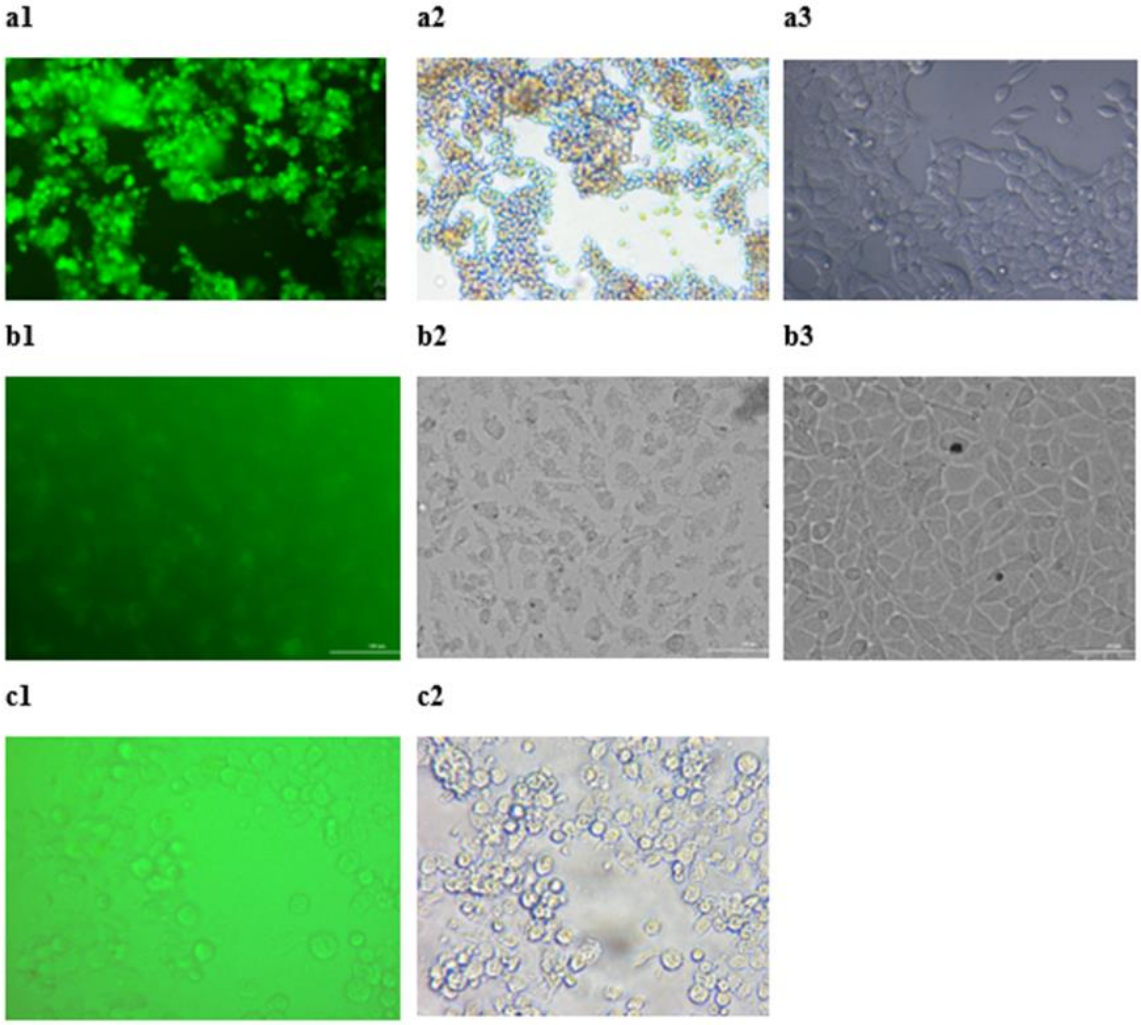

Fig II: Optical and fluorescence microscopy figures. (a) Infected HEK293 cells with Ad scDEC-CEA-GFP: (a1) GFP expression were seen in all HEK293 cells with fluorescence microscopy and (a2) Ad CPE was appeared under optical microscopy versus (a3) uninfected HEK293 cells. (b) Ad scDEC-CEA-GFP infectivity test: (b1) GFP expression were seen in L929 cells but it was weaker than GFP expression in HEK293. It indicated Adenoviruses can transduse their DNA to cells other than HEK293. (b2) Optical microscopy of the same field (b3) uninfected L929 cells. (c) Evaluation of the scDEC-CEA-GFP protein attachment to DCs: Attachment of the scDEC-CEA-GFP protein to DCs were evaluated under fluorescence microscope (c1) Green fluorescent proteins were observed by inverted fluorescence microscope at the surface of DCs threated with supernatants of Ad scDEC-CEA-GFP infected HEK293 cells while there was no obviously GFP in other groups. (c2) Optical microscopy of the same field.

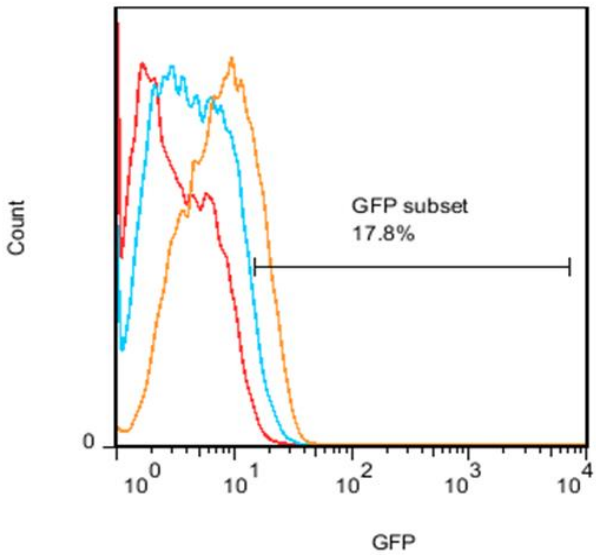

Fig III: Evaluation the attachment of scDEC-CEA-GFP protein to DCs. Untreated and treated DCs with the supernatants of HEK293 cells infected with Ad-CEA-GFP before and after three freezethaw cycles (<1%) (Red). Treated DCs with the supernatants of HEK293 cells infected with Ad-scDEC-CEA-GFP before freezethaw (4.19%) (Blue) and after three freezethaw cycles (17.8%) (Orange). The outcome of this test approved the successful attachment of scDEC to DEC205 receptors on DCs.

and infected with Ad-scDEC-CEA-GFP and Ad-CEA-GFP before and after three freeze-thaw cycles) were added to immature DC cultures and the attachment of fusion proteins to DCs surface was evaluated under fluorescence

microscopy and through the GFP fluorescence measurements by flow cytometry.

Fluorescence microscopy images were shown GFP positive DCs (Fig II). The results of flow cytometry showed that more than 4 and 17 percent of cells were GFP positive in treatments with supernatants of infected HEK293 cells by Ad scDEC-CEA-GFP without and with freeze-thaw cycles respectively while there was no GFP positive cells (<1%) in other groups (untreated DCs, DCs threated with supernatants of uninfected HEK293 cells, DCs threated with supernatants of infected HEK293 cells by Ad-CEA-GFP with and without freeze-thaw cycles). (Fig III). In addition, in DCs threated with supernatants of freeze-thawed infected cells by Ad scDEC-CEA-GFP, a significant increased GFP positive cells were evaluated (17.8%) compared to non-freeze-thaw group (4.19%). The outcome of this test approved successfully attachment of scDEC-CEA polyproteins to DEC205 receptors on DCs.

Discussion

The present work was undertaken to design a new construct for application in studies in the field of cancer treatment in mice. Colorectal cancer is one of the most common cancers in the world (17). Different occurrence frequencies of this cancer has been reported in distinct parts of Iran (18), therefore, it is important to improve and design new methods of colorectal cancer treatment. Combination of common treatment strategies like chemotherapy, radiotherapy and surgery with immunotherapy inclu-ding cancer vaccines have shown promising outcomes (1-3).

One of the key points in preparing an effective vaccine is choosing the appropriate antigens (4). CEA is a glycoprotein of immunoglobulin super family that is expressed during fetal development and also in normal clone epithetlial cells in low level. Overexpression of CEA in colorectal cancers, as well as lung, breast and pancreas cancers has made it a suitable target for cancer vaccines which has led to induction of immune responses (6, 7, 19, 20). Targeting of CEA in clinical trial studies have shown immune responses but those treatment needs modification to improve effective clinical results (21).

Selection a suitable delivery vehicle is another important point in vaccine design (4). Adenoviral vector platform was used in this work because of advantages such as safety, rare pathogenicity to human, ability to infect wide range of dividing and resting cells, potency to accept large size of cDNAs insertion, easily providing mass amount of it and the ability of induction cellular and humoral responses (5, 8). A problem of Adenoviral vector platforms is the presence of anti-vector immunity due to natural infection or the generation of Adenoviral vector immunity upon first immunization (6, 22). To overcome this limitation, it could be helpful to modify the process in which Adenoviral vector inoculation promotes stronger immune responses.

Targeting the antigen to DCs can improve the effectiveness of vaccine and decrease the number of booster doses since DCs are one of the main APCs that have critical role in connecting innate and adaptive immune responses and they can interact with both B cells and T cells, so they have key duty in humoral and cellular responses (9). The results of studies on ex vivo antigen loaded DC based vaccines have been led to entering III phase of clinical trials; however, this approach has some limitation like time consuming and costly vaccine production process and requirement of vaccine construction individually for each patient (9). As a result, in vivo DC targeted vaccines have been generated (9). One of the main focuses of the studies in this context is directing antigen with an antibody single-chain fragment variable (scFv) against to DEC-205, an endocytosis-mediating receptors on the surface of DCs (10- 14). Hence, we built a recombinant Adenoviral vector encoding CEA targeted to DCs by scFv against to DEC-205.

The results of Adenovirus infectivity test and also the outcomes of flow cytometry analysis that evaluated attachment of scDEC-CEA proteins to DCs, have shown reliable conclusions. Showing less GFP positive cells in L929 cells compared to HEK293 may be due to the lack of deleted regions of adenovirus genome

in L929 cells as exist in HEK293 which provide deleted viral proteins in trance (23).
Surveys performed in this study confirmed successful production of an Adenoviral vector expressing scDEC-CEA-GFP but further studies are needed to evaluate the ability of this construct to induce appropriate immune responses in vivo. However, we propose that the Adenoviral vector expressing scDEC-CEA-GFP may be a potential candidate in immunotherapy studies for cancers expressing CEA. In addition, as combination of Adenovirus vector mediated immunotherapy with other treatments achieve better results than either alone (24), using of this construct in combination therapy studies is recommended.

Conclusion

In conclusion, as construction of a suitable product for immunotherapy is a major goal of many research groups, this study led to production of a construct that encoding CEA as an appropriate antigen targeted to DCs by scFv against to DEC-205 in Adenovirus vector platform that can be applied in future in vivo studies in the field of cancer immunotherapy in mice.

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Conflict of Interest

No conflict of interest is declared.

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Ethical Statements

Ethics Committee of Tarbiat Modares University (IR.MODARES.REC 1398.039) approved the study.

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