Cancer Gene Therapy
https://doi.org/10.1038/s41417-018-0038-x

BRIEF COMMUNICATION

Quantification and functional evaluation of CD40L production from the adenovirus vector ONCOS-401

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Received: 7 May 2018 / Revised: 18 May 2018 / Accepted: 24 May 2018

Abstract

Adaptive immunity involves activation of T cells via antigen presentation by antigen presenting cells (APCs) along with the action of co-stimulatory molecules and pattern recognition receptors. Cluster of differentiation 40 (CD40) is one such costimulatory molecule that is expressed on APCs that binds to CD40 ligand (CD40L) on T helper cells and activates a signaling cascade, subsequently resulting in a wide range of immune and inflammatory responses. Considering its important role in regulation of immune response, CD40/40 L has been used for developing antitumor vaccines. In this study, we developed methods for evaluating and quantifying the activity of CD40L expressed from an adenovirus vector ONCOS-401. Our results show that the ONCOS-401 vector produces functional CD40L, which can bind and activate a NF-κB-dependent signaling cascade, leading to secreted embryonic alkaline phosphatase reporter production in HEK293-BLUE cells. In addition, quantification of CD40L production using enzyme-linked immunosorbent assay and HEK-293 BLUE reporter cells showed reproducibly higher recovery of CD40L from ONCOS-401 than from the negative control vector or uninfected cells with consistent inter and intra-assay precision. Thus, a rapid and easy method for quantifying and assessing CD40L production and activity from adenovirus vectors would support the assessment of efficacy of the vector for gene therapy - this was the objective of our study.

Antigen presentation

Antigen presentation, which involves the display of intra and extracellular antigens complexed with major histocompatibility complexes (MHCs) on the surfaces of antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages, and B cells, to effector cells such as T cells (helper and cytotoxic), is key to the development of adaptive immunity and defense against tumors. APCs are classified as professional and non-professional. Those that express MHC class II molecules along with co-stimulatory molecules and pattern recognition receptors are called professional antigen-presenting cells [1], which efficiently internalize antigens either by phagocytosis (macrophages and dendritic cells) or receptor-mediated endocytosis (B cells), process the antigens into peptide fragments, and display them as MHC-bound peptides on their surfaces. The T cell recognizes and interacts with the antigen-class II MHC molecule complex via T cell receptors (TCRs) [2]. Subsequently, an additional co-stimulatory signal is produced by the APCs, leading to activation of the T cell.

CD40

Cluster of differentiation 40 (CD40) is a co-stimulatory protein found on APCs that is required for their activation. The binding of CD40L (CD154) on T_H cells to CD40 activates APCs and induces a variety of downstream effects. CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily and is essential in mediating a wide range of immune and inflammatory responses, including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation [3]. The TNFR-receptor associated factor adaptor proteins TRAF1, TRAF2, TRAF6, and possibly TRAF5 interact with this receptor and act as mediators of the signal transduction [4–8].

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Published online: 30 July 2018

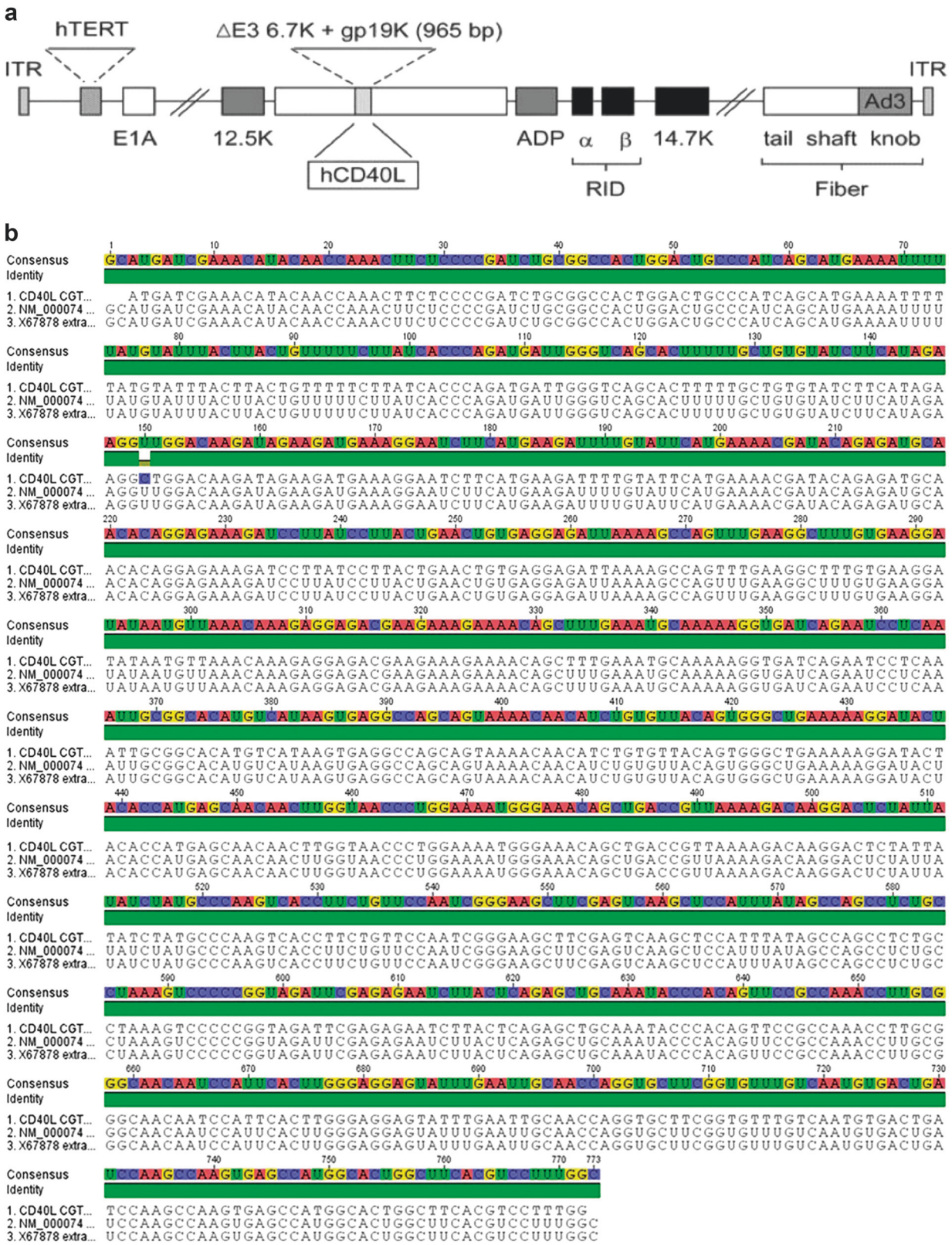

Fig. 1 **a** Structure of the adenovirus ONCOS-401/Ad5/3-hTERT-E1A-hCD40L virus. **b** Genetic analysis of CD40 from ONCOS-401 and two other CD40 sequences (source PubMed, NM_000074 & X67878: *H.sapiens* mRNA for CD40 ligand). Analysis was performed using Genious® 6.0.5

IFN-γ from Th1 type CD4 + T cells is the primary signal for macrophage activation, whereas CD40L on T cells is the secondary signal, which binds to CD40 on the macrophage cell surface. This triggers expression of more CD40 on macrophage surface, which increase the level of activation. The increase in activation induces the production of potent microbicidal substances in the macrophage, including reactive oxygen species and nitric oxide, leading to the destruction of ingested microbes.

B cells can also present antigens to helper T cells. If an activated T cell recognizes the peptide presented by the B cell, the CD40L on the T cell binds to the B cell's CD40 receptor, resulting in B cell activation. As a result of this net stimulation, the B cell can undergo division, antibody isotype switching, and differentiation to plasma cells. The end-result is a B cell that is able to mass-produce specific antibodies against an antigenic target. Indeed, there is little class switching or germinal center formation in CD40-deficient or CD154-deficient mice and immune responses are severely inhibited [9].

CD40 is expressed in a wide range of cell types. In addition to APCs, it is also expressed by endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells [10]. Furthermore, CD40 is also expressed on various types of tumors, including non-Hodgkin's and Hodgkin's lymphomas, myeloma, and some carcinomas of the nasopharynx, bladder, cervix, kidney, and ovary. CD40 is also expressed on B cell precursors in the bone marrow, and studies show that CD40-CD40L interactions may play a role in the control of B cell hematopoiesis [11].

Considering its important role in regulation of immune response, CD40 has been used for developing antitumor vaccines. Parameswaran et al. [12] showed that DCs that have phagocytosed CD40L-expressing apoptotic tumor cells provide new strategies in DC cancer vaccines. Similarly, adenovirus vector-mediated CD40L gene therapy is effective for metastatic liver and colon cancer in rats [13–15], prostate cancer in mouse [16], and CD40-positive bladder, ovary, and cervical carcinomas [14]. In fact, adenovirus-expressing CD40L has been used in first phase I/IIa trials for treating human bladder carcinoma [17]. Adenoviral vectors have been further modified to change the tropism of the virus by incorporating three different proteins, namely, the Ad serotype 5 fiber, phage T4 fibritin, and the human CD40 ligand (CD40L). CD40L retains its functional tertiary structure upon incorporation into this chimera and allows the virus to use CD40 as a surrogate receptor for entry in CD40-positive DCs and tumor cells with high efficiency, which allows genetic immunization and targeted destruction of tumors [18]. Thus, rapid and easy methods for quantifying and assessing CD40L production and activity from adenovirus vectors is critical for estimating the efficacy of the vector for gene therapy.

Assays for quantification and functional evaluation of adenovirus-CD40L

ONCOS-401

In this study, we developed methods for quantifying and evaluating the function of a transgene encoding human CD40L inserted into the adenovirus CONCOS-401. Adenovirus ONCOS-401 (Ad5/3-hTERT-hCD40L i.e., CGTG-401) is a genetically modified micro-organism with four genetic modifications (Fig. 1a), namely, insertion of the human TERT promoter (hTERT) at nucleotides 386-680, human CD40L (hCD40L) at nucleotides 28583-29352, and tail shaft knob 3 at nucleotides 32209-32602. hCD40 L harbors a point mutation at position 150, which changes the codon from CUG to UUG, both of which encode leucine (Fig. 1b), and therefore does not change the amino acid sequence.

Qualitative and quantitative assay for detecting hCD40L

Qualitative analysis of hCD40L production was performed using HEK293-BLUE cells, which stably expresses the human CD40 gene and an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) construct. Binding of ONCOS-401-expressed hCD40L to the CD40 receptor on HEK293-BLUE cells stimulates the TRAF6-mediated signal transduction pathway, which activates NF-κB and leads to SEAP production. SEAP levels are monitored using a Quanti-BLUE assay. Briefly, A549 cells were infected with either ONCOS-401 or ONCOS-102 [19] viruses (negative control, oncolytic adenovirus expressing granulocyte-macrophage colony-stimulating factor ((GM-CSF)) (50 ICC units/cell calculated based on ICC) and incubated for 48 h at 37 °C in the presence of 5% CO_2. A non-infected sample was used as control. Cell supernatants were collected after 48 h, centrifuged at 300×*g* for 5 min, and filtered using 0.22 µm and 0.02 µm filters. Two hundred microliters of each supernatant (ONCOS-401, ONCOS-102, and uninfected cells) were added to 180 µL HEK293-BLUE cells in 96-well plate and incubated for 24 h (maximum) at 37 °C in the presence of 5% CO_2. Recombinant hCD40L (Invivogen, catalog no. rhcd40I) was used as a positive control and all samples were tested either in duplicate or triplicate. SEAP was detected using the SEAP detection kit (Invivogen, catalog no. repqb2) following the manufacturer's instructions. Briefly, 40 µL supernatant of the HEK293-BLUE cells was mixed with 160 µL Quanti BLUE media in a 96 well plate, incubated for 1 h at 37 °C, and the absorbance was measured at 655 nm using a microplate reader.

After subtracting the absorbance of the blank (non-infected) sample, the mean absorbance values were determined for ONCOS-401 and data were plotted using Microsoft Excel. A standard curve was plotted using the

absorbance values of the different concentrations of the recombinant CD40L positive control, and CD40 L concentrations were calculated using regression analysis-Logest. Sample recovery was defined as the ability to correctly quantitate known concentrations of CD40L in a representative sample matrix. For the HEK293-BLUE quantitative assay, recovery was evaluated by spiking 20 µL of 0.416 µg/mL and 0.208 µg/mL with 20 µL of tested samples. The percentage sample recovery is calculated as the total measured CD40L concentration divided by the sum of the spiked plus test sample concentrations. Acceptable recovery was in the range of 80–120%.

The percentage spike recovery was calculated using following equation:

$$\text{Rec}.(\%) = \frac{\text{concentration measured from spiked sample}}{\text{known concentration of sample} + \text{know concentration of spike}} \times 100\%$$

The percentage recovery of the standard was calculated using following equation:

$$\text{Rec.}(\%) = \frac{(\text{calculated concentration} \times 100\%)}{\text{nom. concentration}}$$

Precision, which was defined as the percentage coefficient of variation (%CV) was calculated by dividing standard deviation by the mean for a number of replicate determinations. Intra and inter assay variations were calculated.

We demonstrated (n=3 experiments) that the HEK293-BLUE qualitative assay provides logical and reproducible results (intra & inter assay precision) and can be used to determine the functionality of the transgene encoding CD40L (Fig. 1). The response of HEK293-BLUE CD40 cells to ONCOS-401 CD40L was 8.73–11.8 times higher than that of the negative control supernatant (from non-infected cells) and 9.46–10.98 higher than that of the ONCOS-102 supernatant. Intra assay precision (ONCOS-401/supernatant & ONCOS-401/ONCOS-102) was 2 −6.12%, whereas inter assay precision of ONCOS-401/supernatant was 11.8% and that of ONCOS-401/ONOCS-102 was 8.66%. The results fulfill the criteria of CV ≤ 20%. The HEK-BLUE quantitative assay showed reproducible results. Inter assay precision for calculated CD40L concentration was 9.32%, whereas the same for standard CD40L was 3.3% and that for spiked samples was 9.9% (Table 1). Moreover, the assay provided reproducible and constant values of CD40L produced by ONCOS-401. The results indicate that the assay can be used for the quantification of the CD40L in culture supernatant

Enzyme-linked immunosorbent assay (ELISA) for quantifying CD40L production

An ELISA kit for hCD40L was used per manufacturer's instructions (Abcam, ab99991). Briefly, an antibody

Table 1 Results of HEK293-BLUE quantitative assay and ELISA

			HEK-BLUE quantitative assay			ELISA assay	
Study	Conc. of CD40L (µg/ml)	% CV intra assay precision-samples (OD)	% Rec. of standard CD40L	% Rec. of spiked samples	Conc. of CD40L (µg/ml)	% CV intra assay precision-samples (OD)	% Rec. of standard CD40L
I	0.2	0.17	102	103.1	0.1	6.9	99.9
II	0.2	5.3	102.2	103.3	0.1	10	93.8
II	0.2	0.18	103	99,4	0.12	5.6	100

%CV inter assay precision

Study	Conc. of CD40L (µg/ml)	Samples (OD)	% Rec. of standard CD40L	% Rec. of spiked samples	Conc. of CD40L (µg/ml)	Samples (OD)	% Rec. of standard CD40L	% Rec. of spiked samples
All studies	9,32%	7%	3.3%	9.9%	6.2%	18.2%	4%	8.9%

specific for hCD40L (capture antibody) was first coated on a 96-well plate. Standards and samples were pipetted into the wells and CD40L from culture supernatant bound to the immobilized antibody. The wells are washed and a biotinylated anti-hCD40L antibody (detection antibody) was added. After washing away the unbound biotinylated antibody, horse radish peroxidase (HRP)-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells, and color development was monitored at 450 nm, which was proportional to the amount of bound CD40L. The mean absorbance for each set of standards and samples were calculated after subtracting the absorbance of the negative control (uninfected cell supernatant). The standard curve was plotted immediately after stopping the reaction, with standard concentration on the *y*-axis and absorbance on the *x*-axis, and the best-fit straight line was drawn through the standard points (polynomial correlation). ELISA accuracy was evaluated by spiking 100 µL of the 666.7 pg/mL standard sample with 100 µL test sample.

Reproducible results (intra and inter assay precision) were obtained using ELISA. Inter assay variation for calculated CD40L concentration was 6.2% and 0.1−0.12 µg/mL CD40L was obtained. Standard recovery was in the range of 93.8-100% and inter assay precision of standard protein value was 4%. Recovery of spiked samples were in the range of 107–121.5% and inter assay precision was 8.9% (Table 1). Standard curves presented high value of coefficient of determination (R^2). Overall, the results indicate that the assay can be used for quantification of CD40L in culture supernatants.

Conclusions

In this study, we demonstrated that the HEK293-BLUE assay can be used for determination of the biological function of CD40L expressed from a recombinant virus vector, thus can be used as a tool to monitor the quantity and functionality of the protein in preclinical and clinical settings, including process development for the vector production. Both HEK293-BLUE and ELISA could be used for accurate quantification of secreted CD40L levels although the mechanisms of action of the assays are different. However, technically, the HEK293-BLUE assay is more cost-effective than ELISA. An additional benefit of the HEK293-BLUE assay is that it can be used for both qualitative and quantitative analysis of CD40L activity, however the assay is more complex over simple ELISA technique and requires cell culture in vitro.

Acknowledgements We thank Antti Vuolanto for the help and assistance with the method development.

Compliance with ethical standards

Conflict of interest LK, ASM, MJ are employees and/or shareholders in Targovax Oy in Finland and Targovax ASA in Norway.

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