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Surface display of vascular endothelial growth factor receptor-2 specific nanobody on 293T cells – A potential targeting moiety for lentiviral vector-based cancer therapy

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Abstract

Despite the promising features of lentiviral vectors for cancer gene therapy, tumor targeting remains a major challenge. Incorporation of the specific targeting moieties on the surface of lentiviral particles has turned out to be a major strategy in developing targeted vectors. Herein, we report efficient surface display of a nanobody against vascular endothelial growth factor receptor-2 (VEGFR2), an important angiogenesis and tumor marker, on 293T cells for the final aim of tumor-targeting by lentiviral vectors. To this end, Coding sequence of VEGFR2-specific nanobody was PCR amplified and cloned into pDisplay plasmid. Construction of the final recombinant plasmid encoding VEGFR2-specific nanobody was confirmed. Western blotting analysis for the expression of the recombinant construct (pDis-Nb) in 293T cells revealed a single band around 22 kDa corresponding to the expected size of the recombinant nanobody. Flow cytometric analysis for detection of the membrane-bound form of nanobody indicated efficient display of the nanobody (around 94%) on the surface of the transfected 293T cells. Promising results of this study for efficient expression and cell-surface display of the VEGFR2-specific nanobody (as a major step in production of a targeted lentiviral vectors) indicated the possibility of construction of lentiviral vectors targeted with this nanobody moiety.

Keywords: Nanobody, VEGFR2, Lentiviral vectors, targeted therapy

1. Introduction

Cancer, the abnormal cell proliferation to induce tumorigenesis, is caused by mutations in the key genes governing major cellular functions like cell division and death [1, 2]. Recent advances in molecular biology of cancer and identification of critical genes involved in tumorigenesis, provided the possibility of applying "gene therapy approaches" for the final aim of cancer therapy [3, 4]. Indeed, at present, more than 64% of gene therapy-oriented clinical trials conducting worldwide are designed to exert therapeutic benefits for cancer [5]. The success in gene therapy, however, is mainly based on application of efficient gene delivery systems. In this context, a broad spectrum of non-viral gene delivery tools (such as; liposomes, polymers and cell penetrating peptides) [6] and viral vectors (such as; adenoviruses and adeno-associated viruses, retroviruses specially lentiviruses) [7] have been employed. Being considered as the most efficient gene delivery tools, currently viral vectors are being utilized in majority of gene therapy studies and clinical trials [7-9]. Among viral vectors, retroviruses, RNA viruses with an intermediate conversion of the genome to DNA

following infection of the dividing cells, are preferred tools for gene delivery because of long-term expression of the targeted gene due to the integration into the host genome [10]. Lentiviruses are a subclass of retroviruses which not only infect dividing cells, but also non-dividing and slowly dividing cells as well. This property is of utmost importance in cancer gene therapy, since besides rapidly proliferating cells, tumors typically contain heterogeneous mixtures of slowly dividing and non-dividing cells [11]. Lower genotoxicity and ease of manipulation and production (compared to their other retroviral counterparts) are additional advantages making Lentiviral vectors valuable tools in gene therapy applications [12]. Despite all these benefits, restriction of the infectivity to the target (tumor) cells without affecting non-target (normal) cells remained a major challenging issue in application of lentiviral vectors (and obviously other gene delivery tools) for the final aim of cancer therapy [13]. To address this issue, strategies for enhancing the selective targeting of the lentiviral vectors to the target cells by incorporation of targeting moieties on lentiviral vector surface against tumor cell specific markers, such as natural ligands (like: CD20 [14] and stem cell factor (SCF) [15]), antibodies (like: anti-CD20 [16, 17] and anti-CD3 [18]) or antibody fragments (like: ScFvs) [19, 20] were undertaken. Although, application of antibodies as targeting moieties on lentiviral vectors provided great achievements for targeted tumor therapies, classic and conventional full antibodies are relatively large and complex molecules [21, 22] which their expression on lentiviral vectors require two additional accessory proteins (Igα and Igβ) [18]. To address these limitations application of smaller antibody fragments like ScFvs for targeting of these vectors were considered but their employment encountered other challenging issues like decreased affinity and stability [21, 23]. Discovery of a special group of antibodies in Camelidae species [24] which only consisted of two heavy chains and recognize their cognate antigen via a single N-terminal variable domain (nanobodies), revolutionized the potential medical applications of antibodies and relieved the restrictions of conventional antibodies [22]. In fact, nanobodies are the smallest naturally occurring antigen recognition domains [21], demonstrating interesting characteristics such as; *i)* high affinities in the range of nanomolar to even picomolar concentrations, *ii)* poor immunogenicity, *iii)* ease of gene manipulation, *iv)* high solubility, stability and capability of recognizing cryptic epitopes compared to conventional antibodies [21-23, 25, 26] that makes them an excellent moiety to target nano-sized theranostics (therapeutics and diagnostics) agents to the desired cells [27-30]. In this context, recently, incorporation of antigen-presenting cell-specific nanobodies on the surface of lentiviral vectors was shown to specifically target the resulting viruses to dendritic cells (DCs) and macrophages [31, 32]. Tumor angiogenesis, the growth of new blood vessels to support tumorigenesis, is considered as an important hallmark of cancer [33] and accordingly angiogenesis markers and molecules that could bind specifically to tumor associated endothelial cells might be considered as important candidate targets and targeting moieties respectively, in cancer gene therapy [34]. Vascular endothelial growth factor receptor-2 (VEGFR2) is an angiogenesis associated marker which is preferentially overexpressed on tumor associated endothelial cells [35-37] and thus is an obvious target for cancer targeted therapy. Accordingly, inhibition of tumor vascularization by engineered lymphocytes expressing chimeric antigen receptor (CAR) against VEGFR2 was recently reported [35]. More recently, specific targeting and delivery of therapeutic VEGFR2 antibody-coated nanoparticles to VEGFR2 expressing tumor associated endothelial cells was also described [38, 39]. In addition more than a decade ago, application of conventional antibody-mediated targeting of retroviral vectors towards VEGFR2 expressing cells was reported [40]. Although these previous studies successfully demonstrated the possibility of

antibody application for VEGFR2-targetting but still could be further improved by application of properly characterized nanobodies. However, to our best of knowledge no prior study addressed the application of VEGFR2-specific nanobodies for targeting the viral vectors to date. To address this concern, in the present study, we evaluated the possibility of surface display of a recently described nanobody against VEGFR2 [36, 41] by expression of the membrane-bound form of this nanobody (mNb) on 293T cells (a widely used cell line for production of lentiviral vectors [42]) which is the first and a crucial step in production of a targeted lentiviral vector for the final aim of cancer therapy [43].

2. Materials and Methods

Plasmid Construction

The previously described plasmid encoding the nanobody against VEGFR2 (pHEN6C-3VGR19) [41] was used as the source of nanobody gene in this study. pDisplay plasmid encoding Murine Ig κ-chain leader sequence, Hemagglutinin A epitope tag (HA-tag), *myc* epitope and Platelet-derived growth factor receptor transmembrane domain (PDGFR-TM) was obtained from Invitrogen (Life Technologies, USA) and utilized for expression of the mNb. The cloning steps for construction of pDisplay encoding VEGFR2-specific nanobody (pDis-Nb) are illustrated in Figure 1. Briefly, DNA sequence corresponding to the nanobody (3VGR19) was PCR-amplified from pHEN6C-3VGR19 as template by a pair of primers; *Bgl*II-Nb: CCAGATCTCAGGTGCAGCTGCAGGAG and *Sal*I-Nb: GTGGTCGACTGAGGAGACGGTGACCTG, containing *Bgl*II and *Sal*I restriction sites respectively (recognition sites are underlined). The amplified nanobody fragment was gel purified by GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania), digested *by Bgl*II and *Sal*I enzymes and inserted into the same sites of pDisplay plasmid to produce the recombinant pDis-Nb vector. Resultant plasmid (pDis-Nb) was confirmed by restriction analysis followed by DNA sequencing. All PCR and cloning procedures were performed according to the standard procedures [44].

Cells and transfection procedures

293T (Human Embryonic kidney) cells were obtained from National cell bank of Iran (Pasteur Institute of Iran, Iran). Cells were cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Atocel, Hungary), 100 units/mL penicillin and 100 µg/mL streptomycin (PAA, Austria) in humidified CO2 incubator under the standard aseptic procedure. Transfection of cells was achieved by Turbofect transfection reagent (Thermo scientific, Lithuania) according to the manufacturer's instruction. Briefly, 5×10^5 cells were seeded in each well of a 6-well plate, one night prior to transfection. Cells were transfected with total of 4 µg plasmids (pDis-Nb or pEGFP-N1) and 6 µl of Turbofect transfection reagent for each well. As mock transfection control, 293T cells were transfected with 4 µg of pCDNA3.1(+) (Invitrogen, USA). For evaluation of the transfection efficiency, 293T cells were transfected with 4 µg of pEGFP-N1 (Clontech Laboratories, USA) in a separate well as described above and 48 hours post transfection, GFP expression was analyzed by CyFlow flow cytometer (Partec, Germany).

SDS PAGE and Western blot analysis

48 hours after transfection of 293T cells with pDis-Nb, 1×10^6 cells were harvested and centrifuged at 300×g for 5 minutes. Cell pellets were resuspended in appropriate volume of SDS loading buffer and placed in boiling water for 5 minutes. The suspension was subsequently applied into a 12% SDS-PAGE and separated protein bands were electrotransferred to Polyvinylidene fluoride (PVDF) membrane (Roche, Germany) in wet condition. PVDF

Membrane was blocked by PBS containing 0.1% tween 20 (PBST) and 5% skim milk overnight at 4°C. Primary mouse anti-HA antibody (Cell Signaling, USA) with the final dilution of 1:1000 was added to the membrane and incubated for 1 hour at room temperature. After washing 4 times with PBST, membrane was incubated with secondary anti-mouse HRP conjugated antibody (Cell Signaling, USA) with the final dilution of 1:3000 for 1 hour at room temperature. Subsequently membrane was washed with PBST and Protein bands were visualized by Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences, UK) and Kodak image station (Kodak, USA).

Cell surface expression Analysis by flow cytometry

48 hours after transfection of 293T cells with pDis-Nb, cells were dissociated by 10 mM PBS-EDTA. Around 1×10^6 cells were washed with staining buffer (PBS containing 0.5% BSA), incubated with 100 µl staining buffer containing primary monoclonal mouse anti-HA tag antibody (Cell Signaling, USA) with the final dilution of 1:50 for 1 hour at room temperature and after extensive washing steps incubated in 100 µl staining buffer containing secondary anti-mouse antibody conjugated with phycoerythrin (PE) (Cell Signaling, USA) with the final dilution of 1:500 for 30 minutes at room temperature. Finally cells were washed with PBS and analyzed by flow cytometer. As negative control, mock transfected cells (cells transfected with pCDNA3.1(+)) were subjected to the same procedure followed by flow cytometry.

3. Results

Construction of pDis-Nb plasmid for expression of the mNb

The PCR-amplification of VEGFR2 nanobody gene from pHEN6C-3VGR19 vector resulted in a PCR product of 389 bp which was in accordance with expected size (Fig. 2A). As shown in Fig.1, our cloning strategy (cloning VEGFR2-specific nanobody gene into the *Bgl*II and *Sal*I recognition sites of pDisplay) located the target gene in the same reading frame with signal peptide, HA-tag, *myc* epitope (*myc* tag) and PDGFR-TM. Therefore, expression from this open reading frame (ORF) should produce mNb harboring the above mentioned tags. The authenticity of the recombinant pDis-Nb was confirmed via restriction analysis using pair of *Hin*dIII/*Sal*I enzymes which resulted in two expected DNA band of 5125 bp and 560 bp (Fig. 2B) followed by sequencing analyses (data not shown).

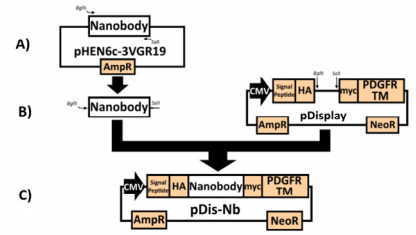

Figure 1. Construction steps of pDis-Nb; A) Amplification of nanobody coding sequence from pHEN6C-3VGR19 by primer pairs containing *Bgl*II and *Sal*I. B) Double digestion of Amplified PCR product and pDisplay with *Bgl*II and *Sal*I. C) Cloning PCR product into linearized pDisplay resulting in construction of pDis-Nb. CMV: Human cytomegalovirus (CMV) immediate-early promoter/enhancer, HA: HemagglutininA epitope tag sequence, myc: *myc* epitope sequence, PDGFR TM: Platelet-derived growth factor receptor transmembrane domain, AmpR: Ampicillin resistance gene, KanR: Kanamycin resistance gene

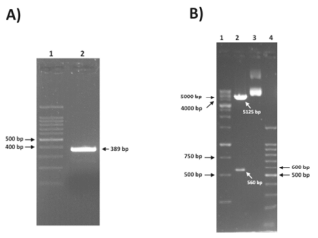

Figure 2. Agarose gel electrophoresis of A) PCR amplification of nanobody coding sequence; lane 1: DNA marker, lane 2 PCR product of nanobody from pHEN6C-3VGR19 (389 bp), B) Double digestion of pDis-Nb with *Hin*dIII and *Sal*I restriction enzymes (5125 bp and 560 bp): lane 1: DNA marker, lane 2: Double digested pDis-Nb, lane 3: undigested pDis-Nb, lane 4: DNA marker.

Analysis of the VEGFR2-specific mNb expression by 293T cells via western blotting

To primarily detect the recombinant protein resulted from the expression of pDis-Nb (VEGFR2-specific mNb), Western blot was performed using anti HA-tag antibody [45]. Western blot analysis of 293T cells expressing pDis-Nb revealed a band of about 22 kDa which was the same as calculated size of this mNb (approximately 21.9 kDa) while no band was observed in mock transfected cells (Fig. 3).

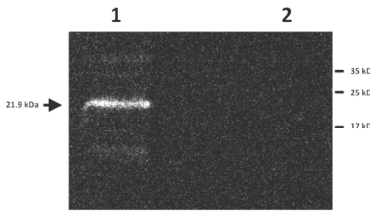

Figure 3. Evaluation of mNb expression by western blot; lane 1: 293T cells transfected with pDis-Nb, lane 2: mock transfected 293T cells

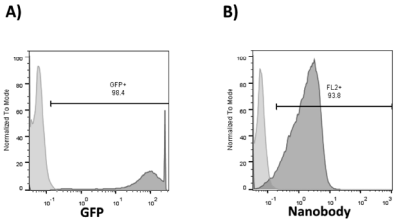

Figure 4. Flow cytometric evaluation of A) transfection efficiency of 293T cells transfected with EGFP expressing plasmid (red area) compared with mock transfect cells (blue area) which is around 98% for EGFP expressing cells and B) presence of membrane bound form of nanobody on the surface of 293T, 48 hours after transfection with pDis-Nb (red area) in contrast with the mock transfected cells (blue area) which is around 94%.

Flow cytometric analysis

Flow cytometric analysis of 293T cells transfected with pEGFP-N1 showed that more than 98% of cells expressed EGFP (Fig. 4A). This result confirmed the expected high transfection needed for the aim of this study. To evaluate the presence of mNb on the surface of pDis-Nb transfected 293T cells, cell surface staining was performed using anti-HA antibody [45] and analyzed by flow cytometry. As shown in Fig. 4B and in accordance with results obtained for pEGFP-N1 (Fig. 4A) around 94% of the cells were positive for the presence of mNb on their surface in comparison to mock transfected cells which was associated with approximately 35 fold increase in geometric mean fluorescence intensity (Fig. 4B). This result indicated the efficient surface display of VEGFR2-specific mNb on 293T cells which might be required for subsequent steps in production of a targeted lentiviral vector.

4. Discussion

Development of lentiviral vectors which could specifically deliver genes to the desired and target cells could improve safety and efficacy of this important group of viral vectors for the aim of gene therapy. In this regard, risk of insertional mutagenesis would be restricted to a specific subset of cells which have been transduced by targeted lentiviral vectors [46]. Furthermore, by overcoming the nonspecific delivery of lentiviral vectors to other cells, the efficacious dose of vector for target cells would be increased while adverse side effects resulting from expression of transgene in unintended cells would be alleviated [12, 47]. Lentiviral particles, due to their promiscuous nature, can incorporate heterologous membrane proteins on their surface by at least two general (active and passive) mechanisms [48-51]. Active model requires direct or indirect interaction between the cytoplasmic tail of heterologous proteins and viral core proteins, while in passive model abundant availability and absence of incompatibility between the cytoplasmic tail and viral core determine the incorporation [52].

In the present study we employed pDisplay vector to display the desired protein on cell surface of 293T cells which is the first and one of the crucial steps in production of a targeted

lentiviral vector for cancer therapy [43]. pDisplay is a commercial plasmid used in several studies to both direct different proteins to the cell surface [53, 54] and to display heterologous proteins on lentiviral vectors. In this context, recently this plasmid was successfully employed to display the designed ankyrin repeat proteins (DARPins) against Her2/neu (a breast tumor marker) on lentiviral vector surface to redirect these viruses to Her2/neu expressing cells [45]. This incorporation might be attributed to the passive mechanism as the membrane proteins resulting from pDisplay contain short cytoplasmic tails of 8 amino acids which decrease the possibility of incompatibility in packaging process. In parallel, recently the feasibility of pDisplay as a platform to display nanobodies against antigen-presenting cells on lentiviral vectors has been also demonstrated in two separate studies [31, 32]. In an early attempt to redirect retroviral vectors to VEGFR2 expressing cells, IgG-binding motifs were inserted into the envelope glycoproteins of retroviral vectors and successfully transduced target cells in the presence of antibody against this receptor in vitro [40]. However this targeting strategy might not find in vivo applications where the serum antibodies (IgGs) compete with the targeting antibody [55]. This disadvantage might be clearly relieved by incorporation of VEGFR2-targeting ligands directly on retroviral (including lentiviral) vectors surfaces. Accordingly and considering the mentioned advantages of nanobodies over conventional antibodies and antibody fragments, in the present study, we aimed to display a VEGFR2 specific nanobody on 293T cells for the final aim of production of a lentiviral vector equipped directly with a suitable targeting moiety (VEGFR2 targeting nanobody) for cancer therapy. To this end, we amplified the sequence of a VEGFR2 specific nanobody (from pHEN6C-3VGR19) which was previously reported to have high affinity to VEGFR2 [41]. Gel electrophoresis of PCR product showed that the length of amplicon was in accordance with the expected size (Fig. 2A) [41]. The amplified fragment was then cloned into pDisplay and the final plasmid (pDis-Nb) was confirmed by sequencing). In the next step pDis-Nb was transfected into 293T cells which are one of the most common cell lines in lentiviral vector production [42]. Flow cytometric analysis of cells transfected by pEGFPN1 vector (EGFP expressing plasmid) indicated 98% transfection efficiency and ensured the high rate of transfection required for our final experimental aim. Expression of pDis-Nb was analyzed by Western blotting using anti HA-tag antibody. Anti HA-tag antibody has been previously used in Western blotting to detect the expression of pDisplay-derived proteins [56]. The results showed the expression of an approximately 22 kDa protein which was in accordance with the expected size of the protein (~13.5 kDa) [41] with addition of vector derived sequences (peptide tags and PDGR-TM of pDisplay vector which are collectively around ~8.5 kDa). As mentioned before, expression of the desired membrane protein on the surface of virus producing cells is an important step for the membrane protein to be incorporated onto lentiviral vector surface especially in passive mechanism. To check whether nanobody could be transported to the cell surface, we stained the surface of transfected 293T cells with anti HA-tag antibody. Anti HA-tag antibody has been previously used to detect the surface expression of pDisplay-derived proteins [45]. It is worth mentioning that, pDisplay is designed in such a way that without an insert in proper reading frame, would not express PDGFR-TM and the resulting protein would not be anchored to the cell surface. Flow cytometric analysis demonstrated the presence of mNb on around 94% of the transfected cells assuring the high cell surface expression of the heterologous protein which might be required for incorporation onto lentiviral vector surface especially in passive mechanism.

5. Conclusions

In summary, to the best of our knowledge, this study is the first report on displaying of VEGFR2 specific nanobody on the surface of 293T cells. Our results indicated high surface expression rates for this protein indicating the great potential of the success for the final aim of this study.

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