European Journal of Pharmaceutical Sciences 49 (2013) 550–555

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Contribution of syndecans to lipoplex-mediated gene delivery

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ARTICLE INFO

Article history:
Received 10 December 2012
Received in revised form 26 April 2013
Accepted 23 May 2013
Available online 1 June 2013

Keywords:
Gene delivery
Liposome
DMRIE-C
Heparan sulfate proteoglycans
Syndecan
Macropinocytosis

ABSTRACT

The long awaited breakthrough of gene therapy significantly depends on the in vivo efficiency of targeted intracellular delivery. Hidden details of cellular uptake present a great hurdle for non-viral gene delivery with liposomes. Growing scientific evidence supports the involvement of polyanionic cell surface carbohydrates in cellular internalization of cationic liposomes. Syndecans, a highly conserved family of transmembrane heparan sulfate proteoglycans serve attachment sites for great variety of cationic ligands including growth factors, cytokines and even parasites. In the present study we quantitatively measured the contribution of various syndecan isoforms to liposome-mediated gene transfer. The obtained data show the superiority of syndecan-4, the ubiquitously expressed isoform of the syndecan family, in cellular uptake of liposomes. Applied mutational analysis demonstrated that gene delivery could be abolished by mutating the glycosaminoglycan attachment site of syndecans, highlighting the importance of polyanionic heparan sulfate side chains in the attachment of cationic liposomes. Blocking sulfation of syndecans also diminished gene delivery, a finding that confirms the essential role of polyanionic charges in binding cationic liposomes. Mutating other parts of the syndecan extracellular domain, including the cell-binding domain, had clearly smaller effect on liposome internalization. Mutational analyses also revealed that superiority of syndecan-4 in liposome-mediated gene delivery is significantly influenced by its cytoplasmic domain that orchestrates signaling pathways leading to macropinocytosis. In summary our study present a mechanistic insight into syndecan-mediated macropinocytic uptake of lipoplexes and highlights syndecan-4 as a superior target for cationic liposomes.

1. Introduction

The future evolution of gene therapy depends significantly on the efficient and selective delivery of bioactive oligonucleotides (DNA, RNA) into the cells. While viral vectors provide the most efficient method for gene delivery, in terms of biological inertness, health risks, synthetic carrier systems such as cationic liposomes still offer considerable advantages over viral vectors (Halama et al., 2009; Zuhorn et al., 2002). Cationic liposomes complex with DNA to form condensed structures called lipoplexes (Elouahabi and Ruysschaert, 2005; Even-Chen and Barenholz, 2000). Compared to viral vectors, transfection efficiency of lipoplexes is still relatively low (Halama et al., 2009; Pack et al., 2005). Efforts to increase efficacy of cationic liposome mediated gene delivery are greatly hampered by the unknown details of cellular internalization. In spite of early studies suggesting cationic lipoplexes enter the cells via fusion with the plasma membrane, lipid mixing assays could not reveal correlation between fusogenicity of lipoplexes and their transfection efficiency (Khalil et al., 2006; Midoux et al., 2008). Later it has been shown that cationic lipoplexes are endocytosed into the cells after electrostatic binding to the negatively charged heparan sulfate proteoglycans (HSPGs) of the cell surface (Poon and Gariepy, 2007), while a recent study demonstrated that nonviral gene delivery vectors use syndecan-dependent transport mechanisms during cellular internalization (ur Rehman et al., 2012).

HSPGs on cell surface are abundant molecules consisting of a protein core with covalently attached heparan sulfate (HS) chains.

Abbreviations: Hs, heparan sulfate; HSPGs, heparan sulfate proteoglycans; SDC, syndecan; CBD, cell-binding domain; CPPs, cell-penetrating peptides; Si, signal sequence.

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http://dx.doi.org/10.1016/j.ejps.2013.05.022

The two major families of HSPGs are the glypicans and the syndecans (Iozzo, 2001; Tumova et al., 2000). Glypicans, predominantly expressed in the central nervous system, are GPI-anchored extrinsic membrane proteins, while the highly conserved four-member family of syndecans (SDCs) are transmembrane core proteins (Bernfield et al., 1999). SDCs are made of a cytoplasmic domain, a highly conserved single-span transmembrane domain, and a divergent extracellular domain with glycosaminoglycan attachment sites for three to five HS or chondroitin sulfate chains (Bernfield et al., 1992; Kokenyesi and Bernfield, 1994). The extracellular domain (ectodomain) of syndecan-4 (SDC4) also contain a cell-binding domain (CBD) mediating attachment to other cells (McFall and Rapraeger, 1997). Contrary to their conserved ectodomains, SDCs show distinct temporal and spatial expression patterns hence are likely to function specifically in vivo (Letoha et al., 2010). In adult tissues, SDC1 is predominantly expressed by epithelial and plasma cells, SDC2 by endothelial and mesenchymal cells. SDC3 expression is mostly restricted to neural cells, while SDC4 is expressed ubiquitously (Bernfield et al., 1992). The presence of polysulfated HS chains on SDC ectodomains enables and facilitates interactions with a large number of cationic ligands, including viruses, growth factors, extracellular matrix proteins (Bishop et al., 2007; Park et al., 2000). SDC1 has already been reported to enhance lipoplex-mediated gene delivery (Mounkes et al., 1998). A recent study has also shown that attaching the laminin-derived peptide AG73, a ligand for SDCs, to polyethylene glycol liposomes can enhance gene transfer in SDC2 overexpressing cancer cells (Negishi et al., 2013, 2010a,b). On the other hand, the SDC2 ectodomain has been shown to inhibit gene delivery mediated by the cationic polymer polyethyleneimine, a frequently utilized polyplex (Paris et al., 2008). In our latest paper, we have already shown, that SDCs, especially SDC4 significantly increases cellular uptake of cell-penetrating peptides (Letoha et al., 2010). Cell-penetrating peptides (CPPs) are short cationic peptides capable of transporting conjugated bioactive compounds including proteins and oligonucleotides intracellularly (Fonseca et al., 2009; Heitz et al., 2009; Vives et al., 2008). Cellular uptake of CPPs and lipoplexes shares common similarities (Hoekstra et al., 2007; Poon and Gariepy, 2007; Rawat et al., 2007). Therefore it was high time to the contribution of the various SDC isoforms to cationic liposome-mediated gene delivery. Using DMRIE-C as a model cationic liposome our approach included the measurement of lipoplex internalization and gene transfer on cell-lines overexpressing the most common SDC isoforms. Structural mutation of the SDC core protein revealed the role various SDC domains play in cellular uptake of lipoplexes. Applied confocal microscopic and quantitative fluorometric methods enabled us to shed light on yet unknown details of lipoplex internalization thus helping future attempts of targeted gene delivery.

2. Materials and methods

2.1. SDC vector constructs

Full-length SDC1, SDC2, SDC4 and deletion mutants were amplified and subcloned in the mammalian expression plasmid obtained from Clontech (pcDNA3, pEGFP). The SDC-chimeras were constructed by inserting green fluorescent proteins (GFP) into the juxtamembrane region of the extracellular segment. The signals were kept in all cases to orient the proteins into the membrane.

2.2. Cell culture and transfection

K562 cells permanently expressing human SDC1, SDC2, SDC4 or the truncated forms of SDC1 and SDC4 were transfected using human SDC DNA constructs as described previously (Letoha et al., 2010). Expression of SDC1, SDC2 and SDC4 in K562 transfectants was measured with a FACScan (Becton Dickinson) using specific antibodies (anti-human SDC1 [Clone 359103], anti-human SDC2 [Clone 305515], and anti-human SDC4) against the studied SDC isoform according to the recommendations of the manufacturer (R&D Systems, Inc.). In the case of GFP-tagged SDC mutants, expression was also analyzed with flow cytometry measuring fluorescence intensities of the GFP tags. Thus clones with equal amount of SDC expression were selected and used to quantify lipoplex-mediated gene delivery.

2.3. Luciferase reporter gene assays

To examine lipoplex-mediated gene delivery, SDC overexpressing cell lines were seeded (0.1 million cells/well) in twenty-four-well plates and transfected with 3.3 μl/well DMRIE-C (Invitrogen) containing 0.66 μg/well pDNA expressing a "humanized" secreted Gaussia Luciferase as reporter gene (pCMVGLuc, 5,7Kbp [pGLuc], New England Biolabs, USA) according to the manufacturers' instructions. Forty-eight hours later, the activity of luciferase expression was quantified with a commercial kit (Gaussia Luciferase Assay kit, New England Biolabs, USA) according to the manufacturer's protocol. The activity is indicated as relative light units (RLU) per mg protein.

2.4. Cell viability

Effect of lipoplex-mediated gene delivery on the viability of cells was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. One day before transfection, K562 cells and SDC clones were seeded onto 96-well culture plates (100,000 cells/well). Volume of 200 μl of the complexes were added to each well and incubated for 4 h. After the incubation the cells were washed and fresh medium was added and incubated for further 48 h. Finally, MTT assay was performed as described previously (Nomani et al., 2010).

2.5. Flow cytometry

K562 and SDC clones were transfected with pEGFP using DMRIE-C reagent (Gibco) as described previously (Kantakamalakul et al., 2003). Forty-eight-hour post-transfection, the expression of EGFP in K562 cells and SDC transfectants was examined with flow cytometry (Becton Dickinson). A minimum of 10,000 events per sample was analyzed. Viability of cells was determined by using appropriate gating to exclude dead cells, debris, and aggregates in a forward-scatter-against-side-scatter plot.

2.6. Confocal laser scanning microscopy

Plasmid DNA (pGLuc) and the fluorescent DNA-intercalators (YOYO-1 or YOYO-3 [Invitrogen]) were mixed at a ratio of 1 YOYO molecule per 75 base pairs (0.8 mol%) and were allowed to complex for 10 min at room temperature. YOYO-labeled DNA was then used to prepare DMRIE-C/DNA complexes exactly as outlined in the experimental procedures above. Internalization of the fluorescently labeled plasmid DNA using DMRIE-C into K562 cells and SDC transfectants was visualized by confocal laser scanning microscopy. Cells were grown on poly-D-lysine-coated glass-bottom 35-mm culture dishes (MatTek Corp.). After 24 h, the cells were preincubated in RPMI 1640 medium (supplemented with 10% FCS) at 37 °C for 30 min before incubation with the fluorescently labeled DMRIE-C/DNA complexes. After 60 min at 37 °C, the cells were rinsed three times with ice-cold PBS and nuclei were stained with 2 μg/ml Hoechst 33,258 (Sigma) for 10 min. Distribution of