METHODS OF PROTEIN DELIVERY INTO MAMMALIAN CELLS FOR GENE THERAPY AND GENETIC STUDIES

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Summary. Methods, lying on different principles for introducing proteins into mammalian cells were compared in the present study. Some of them rely on non-covalent complex formation (Chariot), osmotic lysisis of pinocytic vesicles (Influx pinocytic cell-loading reagent), electric power (electroporation), lipid-based delivery system (Bioporter), microinjection, small protein transduction domains (PTDs) from viral proteins, bacterial secretion system type III (T3SS). Thus, the delivery by many of the compared methods can give a sufficient number of uniformly loaded cells for different studies including transcription and translation, cell cycle regulation, control of apoptosis, oncogenesis and gene therapy.

Key words: *protein delivery, mammalian cells, gene therapy, electroporation, bioporter, microinjection, nanoparticles, cell-penetrating peptides, pinocytic vesicles*

To verify whether the functional activities observed in vitro actually occur also in vivo, the protein under study is “inserted” into cultured cells or an animal. The protein delivery is accomplished indirectly by transfecting transcriptionally active DNA into living cells, where the gene is expressed and the protein is produced by the cellular machinery. Several transfection techniques for introducing proteins into mammalian cells, including electroporation, microinjection, hypotonic shock, scrape loading, viral vectors (retroviruses), cationic liposomes, calcium phosphate coprecipitation are time-consuming (1-3 days to express the transfected genes) and can be cytotoxic and physically disruptive to cells, thus compromising the results from physiological studies. The direct introduction of purified preparation of protein into cells by microinjection, electroporation, construction of viral fusion proteins, cationic lipids may be used in cell cycle regulation, control of apoptosis, oncogenesis and transcription regulation studies. The biological functions can be performed immediately after the protein entry into the

cell. The disadvantages of the method are the toxicity to the recipient cells, the non-specificity, the low transfection efficiency and substantial variability.

Bioporter protein delivery reagent (Genlantis) is non-cytotoxic and transports proteins inside the target cells with retaining structure and function. Bioporter forms a single non-covalent transfection complex, uses non-endosomal delivery mechanism and provides high cell viability. Fluorescent molecules, antibodies, dextran sulfate, phycoerythrin-BSA, β-galactosidase, apoptotic proteins granzyme B, caspases 3 and 8, delivered with Bioporter are functional and effective in multiple cell types such as HeLa, HeLa-S3, BHK-21, 293, CHO-K1, NIH 3T3, CV-1, B16-F0, COS-1, K562, COS 7, Jurkat, Ki-Ras 267 β1, HepG2, MDCK. Depending on the physical properties, the protein in hydration medium is captured by association with the liposomal membrane and attached to negatively charged cell surfaces. The liposomes fuse with the plasma membrane, or are endocytosed and fuse with the endosome, releasing the protein into the cytoplasm. Trifluoroacetylated lipopolyamine and dioleoyl phosphatidylethanolamine (BioPorter) were used to form protein-lipid complexes, containing recombinant herpes simplex virus type-1 thymidine kinase or Drosophila melanogaster multisubstrate deoxyribonucleoside kinase. The complexes were imported into human osteosarcoma and Chinese hamster ovary cell lines by endocytosis. Delivered into the cytosol and nucleus, the proteins retained their enzymatic activity. The direct delivery of suicide gene proteins to cells may be an alternative approach to conventional suicide gene therapy strategies [1]. An amphiphile (CholCSper), consisting of cholesterol, linked to carboxy-spermine by a cysteine, and dimerizable upon mild oxidation of thiol to disulfide, was developed and used with DOPE to prepare intracellular protein delivery system for functionally active proteins [2]. The cholesterol affects the density of the complexes formed with proteins and leads to a prolonged protein release in the cytoplasm of cells exposed to protein carrier assemblies [3]. The protein entry is rapid, concentration-dependent and works with difficult cell types. The protein can be denatured and needs to be refolded upon its entry into the cell to regain its biological activity. The physical properties of delivered protein such as charge and hydrophobicity can influence the interaction with cationic lipids that are used for protein delivery [4]. Pro-ject protein transfection reagent (Pierce) uses a cationic lipid with non-covalent nature, is non-cytotoxic and capable to deliver a variety of proteins into numerous cell types, maintaining their functional activity. The liposome:protein complex fuses with the cell membrane or is internalized via an endosome and the protein or macromolecule of interest is released into the cytoplasm free of lipids.

Pulsin (Polyplus transfection) allows to study lethal proteins by controlling the level and time course of protein delivery into the cells. Pulsin is non-toxic and contains a cationic amphiphile that forms non-covalent complexes with proteins and antibodies, internalized via anionic cell-adhesion receptors and released into the cytoplasm. Intracellular proteins with antibodies in living cells can be targeted with-

out fixation [5]. Proteins, peptides, antibodies, Streptococcus TPE B epitope, FITC-labeled anti-alpha-tubulin, R-phycoerythrin (fluorescent protein of 240 kD) were successfully delivered to HeLa cells up to 98%. The delivery of substrate, inhibitor, modulator, or blocking peptides and antibodies into cell allows protein function studies and RNA interference experiments, as well as the development of therapeutic approaches. The Provectin (Imgenex) is a non-cytotoxic lipid-based protein delivery reagent that allows the delivery of proteins, peptides or other bioactive molecules into the cytoplasm of a variety of different adherent and suspension cells.

The efficiency of electroporation is dependent upon the strength of the applied electrical field, the length of the pulses, temperature, composition of the buffered medium. The cells are grown on a glass slide, half of which is coated with electrically conductive, optically transparent, indium-tin oxide. A control on the strength of the electric field achieved peptide introduction into 100% of the cells, causing no detectable disruption of their division cycle. The method is applied for the fluorescent dye Lucifer yellow, causing its penetration into the cells, growth on the conductive half of the slide [6,7]. Membrane-impermeant molecules, such as small fluorescent dyes, large carrier-based dyes (fluorescein-labeled dextran), large macromolecules (antibodies), and metabolic precursors (32P-ATP) were introduced by electroporation into adherent cells with high efficiency. The suitable poration medium includes PBS, PBS-buffered 0.25-3.0 M sucrose, Hepes-buffered sucrose, unbuffered sucrose. The ability to load foreign molecules into adherent cells is used in microscopic approaches, such as fluorescence spectroscopic imaging, as well as in conventional biochemical and physiological techniques [8, 9]. The electroporation was applied to DU145 prostate cancer cells, incubated with GFP-encoded DNA plasmid, either naked or packaged with cationic lipid (Lipofectin), polycationic peptide (salmon protamine) or retroviral vectors (Moloney murine leukemia viruses), and then assayed for gene expression and cell viability. The combination of electroporation with chemicals as cationic lipid, or viral vectors does not improve the gene transfection in vitro [10]. The proteins cytochrome c, granzyme-B, caspase-8, known to activate caspase-family cell death proteases, were introduced into human leukemia and lymphoma cell lines, as well as into freshly isolated lymphocytes and leukemia cells, by electroporation. Fluorochrome-labeled proteins with MW from 15 to 150 kDa were used to evaluate the electroporation efficiency by flow cytometry and to compare the efficiency of protein delivery, using various electroporation conditions. Electroporation of apoptotic proteins into intact lymphoid cells can be used to contrast the status of various caspase activation pathways, related to pathological defects in the regulation of apoptosis, that exist in individual patient specimens [11]. Effect of electroporation in combination with a plant toxin, saporin, was studied using a human lung cancer cell line (PC9) and a pancreatic cancer cell line (ASPC-1). High degree of inhibition of the proliferation was obtained when ASPC-1 cells were electroporated in the presence of saporin

(0.1-1000 ng/ml), in combination with EP (80-90 V, 10 ms, n = 8). PC9 or ASPC-1 tumor-bearing nude mice were treated with electroporation, following the intratumoral injection of saporin (1 mg). Tumor necrosis was observed 24-48 hr after the combination therapy with saporin and electroporation [12]. Electroporation was used to introduce antibodies into cells without affecting the physiological integrity of the cells [13, 14]. Antibodies to pp60c-src were introduced into cultured RASM cells by electroporation, while still attached to tissue culture plates. Angiotensin II-stimulated tyrosine phosphorylation of phospholipase C-gamma 1 was eliminated by the anti-pp60c-src antibodies, and not by anti-mouse IgG or bovine serum albumin. Electroporation of RASM cells with anti-pp60c-src antibodies had no effect on the platelet-derived and growth factor-stimulated tyrosine phosphorylation of PLC-gamma 1 [15]. A protocol for electroporation results in the uptake of detectable amounts of antibodies in 80% of the mammalian cells. As an example, the cell cycle activity (transition from the G1 to S phase), was inhibited by the introduction of monoclonal antibodies against G1-specific cyclin D1 into CV-1 and MCF7 cells. This specific, antibody mediated inhibition of cellular function did not affect the viability of the cells, since they recovered from the inhibition after some time. The method approaches the efficiency of microinjection and permits treatment of the larger number of cells which are required for biochemical analyses [16]. Disadvantage is that electroporation can be inefficient and highly disruptive, causing large scale cell death. Microinjection has the advantage of introducing macromolecules directly into the cell [17].

An alternative method for introducing of peptides into primary lymphocytes and lymphoid cell lines is the cellular import of the third helix of Antennapedia homeodomain protein (pAntp). The pAntp peptide is taken up rapidly into cell cytoplasm and nucleus, where is retained for at least 48 h. The pAntp peptide is not cytotoxic and the efficiency of delivery is up to 95% [18]. The small protein transduction domains (PTDs) from viral proteins, including HIV-1 TAT protein, herpes simplex virus 1 (HSV-1) DNA-binding protein VP22, Drosophila Antennapedia (Antp) homeotic transcription factor, exhibits properties for spontaneous intracellular penetration and can be fused to other macromolecules, peptides or proteins to transport them into cells [19]. The PTDs or signal peptide sequences are composed from 15 to 30 amino acids and mediate protein secretion into the cells. They have a positively charged amino terminus, a central hydrophobic core and a carboxyl-terminal cleavage site recognized by a signal peptidase. A mixture, containing a protein and a carrier reagent includes a helper reagent to enhance the protein delivery efficiency greater than 90%. The surface-mediated protein delivery technique or "reverse protein delivery" provide greater control over the exact sequence, content, amount and nature by pre-selecting, modifying or checking the proteins prior to their introduction into cells. The protein transduction domains are used for delivery of therapeutic proteins into animal models of human neurological disorders such as nerve trauma and ischemia [20]. Tat, fused to heterologous pro-

teins traverses the biological membranes and the blood-brain barrier in protein transduction. TAT-eGFP or GST-eGFP proteins were fed to C. elegans worms, which resulted in specific localization of Tat-eGFP to the epithelial intestinal cells. The method is a tool to analyze the mechanisms of protein transduction and to complement RNAi/KO in epithelial intestinal system combining the potential of bacterial expression system and Tat-mediated transduction technique in living worm [21]. MPG technology uses virus-derived amphipathic peptides that directly interact with nucleic acid cargos to form nanoparticles (150-200 nm), capable of diffusing through plasma membranes and releasing their contents inside the cell. The mechanism of entry is receptor-independent, involves MPG/lipid membrane interactions, avoids the endocytic pathway and prevents endosomal or lysosomal degradation of cargos. Peptide transfection is used for modulation of cell signaling pathways and creation of potential therapeutic agents such as development of siRNAs [22, 23].

CPPs (cell-penetrating peptides) are able to efficiently penetrate cellular lipid bilayers. The major cell CPPs penetratin, Tat and transportan 10 (TP10) deliver cargos, including fluoresceinyl moiety, double stranded DNA and proteins (avidin, streptavidin), liposomes and nanoparticles [24]. The toxicity caused by CPPs depends on peptide concentration, cargo molecule, high efficacy and coupling strategy. M918 internalizes via endocytosis and macropinocytosis, independently from cell surface glycosaminoglycans and is non-toxic. In a splice correction assay, using antisense peptide nucleic acid (PNA), conjugated via a disulphide bridge to M918 (M918-PNA), there was observed a dose-dependent increase in correct splicing with potential to be used as a therapeutic approach for regulating splicing in a variety of diseases [25, 26]. TP10 was evaluated as a delivery vector in different in vitro plasmid delivery assays that can enhance polyethyleneimine mediated transfection at relatively low concentrations and may help to develop future gene delivery systems with reduced toxicity [27]. CPPs are used as vectors for multiple effectors of gene expression such as oligonucleotides for antisense, siRNA, dsDNA applications, for plasmid delivery transfection agents [28]. The advantage of using splice correction for evaluation of CPPs is that the oligonucleotide (ON)-based therapeutics induces a biological response in contrast to traditionally used fluorescently labeled peptides [29]. Several Gram-negative pathogenic bacteria have a conserved and unique protein secretion system type III (T3SS) to deliver bacterial effector proteins into eukaryotic cells to modulate host cellular functions. These bacterial devices are present in both plant and animal pathogenic bacteria and are evolutionarily related to flagellar apparatus. The development of prevention and therapeutic approaches for several infectious diseases can be based on T3SS [30]. T3SS are encoded by bacterial species that are symbiotic or pathogenic for humans, other animals (including insects or nematodes), and plants related to symbiosis and pathogenesis [31].

Entrypep (Inbiolabs) contains a cell membrane permeable polypeptide sequence that crosslinks by thioester bond and delivers peptides, proteins, antibodies, protein complexes, chemically modified proteins, all compounds with -SH group, dye-labelled proteins. The method may be used for visualisation and colocalisation of cell membrane permeable polypeptide sequence-conjugated antibodies and specific ligands, protein-protein interactions in the cell, inhibition or activation of intracellular processes, functional analysis, intracellular effects of chemically modified proteins [32].

Chariot (Active Motif) peptide forms a non-covalent bond, is non-cytotoxic, quickly and efficiently (up to 60-95%) transports biologically active proteins, peptides and antibodies directly into cells. The method is used for functional studies, including delivery of inhibitory proteins, label organelles, screen peptide libraries, determine protein half-lives and transient complementation. The complex dissociates, leaving the macromolecule biologically active and free to proceed to its target organelle. Chariot was used to deliver carcinoma proteins, peptides and antibodies in HeLa cells with up to 95% efficiency, determined by flow cytometry. Protein transduction was performed using Chariot in rat pheochromocytoma PC12 cell line (RCB009) [33].

Nanoparticle made of hydrophilic polysaccharides, chitosan (CS) and glucomannan (GM) can be used for association and delivery of proteins. The release of peptide/protein can be modulated by varying the composition of the system. Two different types of glucomannan (non-phosphorylated Konjac GM (KGM) and phosphorylated GM) exhibited a great capacity for association of the model peptide insulin and immunomodulatory protein P1, reaching 89% association efficiency [34,35]. A targeted proapoptotic anticancer drug delivery system (DDS) contains PEG as a carrier, camptothecin (CPT) as anticancer drug/cell death inducer, a synthetic analogue of luteinizing hormone-releasing hormone (LHRH) peptide as targeting moiety/penetration enhancer, and a synthetic analogue of BCL2 homology 3 domain (BH3) peptide as suppressor of cellular antiapoptotic defense. The design of multicomponent DDS allowed conjugation of one or two copies of each active ingredient (CPT, LHRH, and BH3) to one molecule of PEG carrier thus ensuring highest anticancer efficiency in vitro and in vivo. The ligand-targeted DDS preferentially accumulated in the tumor and allowed the delivery of active ingredients into the cellular cytoplasm and nuclei of cancer cells [36].

The Influx reagent cell-loading method (Invitrogen) is based on the osmotic lysis of pinocytic vesicles for loading water-soluble and polar compounds into live cells, no altering normal cell function [37]. The compounds to be loaded are mixed at high concentration with a hypertonic medium, allowing the material to be carried into the cells via pinocytic vesicles. The cells are then transferred to a hypotonic medium, that resulted in release of the trapped material into the cytosol. The Influx pinocytic cell-loading reagent contains a mixture of sucrose crystals and PEG that allows loading of compounds into cells, grown on coverslips, in suspension and in flasks.

The biology of the cancer can be modulated at protein level by direct cellular introduction of peptides, full-length proteins and functional domains into tumor cells that allow treat diseases such as cancer. The barrier of the cell plasma membrane restricts the intracellular uptake of macromolecules to non-polar and less than 500 Da in size [38].

CONCLUSION

Thus, the delivery by many of these methods, followed by Fluorescence Activated Cell Sorting (FACS) and spectrofluorimetry of the cell population, can give a sufficient number of uniformly loaded cells. This can be important in choosing a method for protein delivery, when the efficiency is important. The efficiency of the selected viable cells is sufficient for functional studies in vivo and in vitro such as transcription and translation, cell cycle regulation, control of apoptosis, oncogenesis and gene therapy.

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