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Chapter 1

Targeted Gene Delivery: Importance of Administration Routes

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Additional information is available at the end of the chapter

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1. Introduction

Gene therapy is a promising approach to treat intractable and refractory diseases at the genetic level. Basically, in gene therapy, target gene expression is induced by delivering foreign genes. Downregulation of target gene expression or gene silencing can also be performed using miRNA, siRNA or shRNA expression vectors [1]. Gene therapy is useful for both genetic and acquired diseases. For genetic diseases, the first clinical trial was performed for adenosine deaminase deficiency in 1990 [2]. Subsequently, numerous clinical trials were carried out for other congenital genetic defects such as familial hypercholesterolemia and cystic fibrosis [3]. Gene therapy clinical trials were also performed for acquired diseases such as cancers, cardiovascular diseases and infectious diseases [3].

There are two strategies to perform gene therapy, that is, *ex vivo* methods and *in vivo* methods. In *ex vivo* gene transfer, once cells are taken from a patient, *in vitro* gene transfer is performed, and then transfected cells are introduced into the patient. Since *ex vivo* gene transfer requires a cell culture facility, the procedure is cumbersome. On the other hand, *in vivo* gene transfer is performed by directly administering genetic medicine into the patient. When foreign genes are administered into systemic circulation as a naked form, they are rapidly taken up by the reticuloendothelial system and degraded by nuclease in the blood [4]; thus, foreign genes themselves are generally inactive in gene transfer. As such, to achieve *in vivo* gene transfer, both viral and non-viral vectors have been utilized. In both cases, the selectivity of transgene expression in target organs/sites/cells would determine the therapeutic outcome. Uncontrolled transgene expression in non-target organs/sites/cells is problematic due to high biological activities of transgene products. Furthermore, undesirable biodistribution of vectors leads to

their loss and vector-dependent side effects. Thus, gene delivery systems that are targeted to specific organs/sites/cells are important for not only efficacy but also safety.

2. Overview of targeted gene delivery

There are several strategies to achieve targeted gene delivery. Among them, modification with a ligand for specific receptors on target cells is a rational approach. Viral vectors natively utilize specific receptors. For example, adenoviral vector serotype 5 utilizes coxsackievirus and adenovirus receptor (CAR) and integrin, which are abundant on mouse hepatocytes [5, 6]. On the other hand, the receptor for adenoviral vector serotype 35 is CD34, which is expressed on human hematopoietic stem cells [7]. As another good example, sugar modification of vectors is useful. Galactosylation of vectors is useful for targeting to hepatocytes via asialoglycoprotein receptors [8], whereas mannosylation is useful for targeting to macrophages [9]. Furthermore, antibodies against cell surface proteins are also a useful tool for targeting. Antibody against transferrin receptors is utilized for targeting to the brain [10, 11].

Activation of vectors by target cell-specific enzymes is also a rational strategy. In most tumor cells, protein kinase Cα (PKCα) is hyper-activated. A cationic polymer having a peptide substrate of PKCα is specifically phosphorylated in tumor cells; subsequently, the polymer is detached from DNA and transgene expression is turned on [12]. As a similar strategy, a polymer having HIV proteinase-cleavable cationic residues has been developed [13].

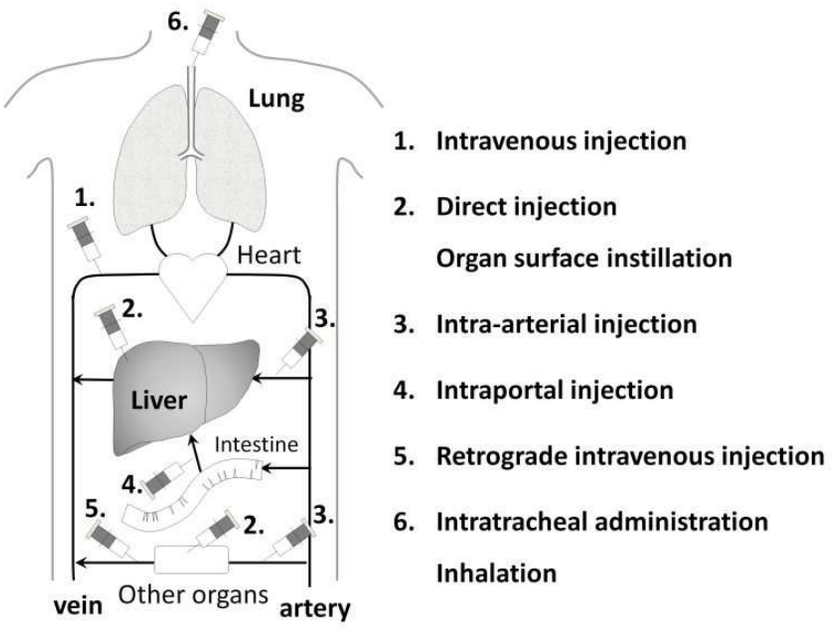

Figure 1. Scheme of administration routes for targeted gene delivery.

To regulate transgene expression in target cells, a tissue-selective promoter can be utilized. For example, albumin promoter and human α1-antitrypsin promoter selectively work in liver

hepatocytes [14]. Tumor-selective promoters such as AFP promoter [15] and CAE promoter [16] are useful to improve tumor-selective transgene expression.

Selection of administration routes is a simple and useful way to control the *in vivo* fate of both viral and non-viral vectors. Selection of administration routes can be combined with other strategies. Depending on the administration routes, accessibilities of vectors to target organs/sites/cells vary significantly. Thus, selection of administration routes is important.

3. Administration routes

Figure 1 shows a schematic representation of administration routes for targeted gene delivery. When target cells are distributed throughout the body, various administration routes can be chosen. Antigen-presenting cells such as macrophages and dendritic cells are good examples. Factors affecting transgene expression, such as interaction with blood components and retention time, are different in each administration route. In addition, transfected cell types are dependent on administration routes. When target cells have polarity, secretion polarity of transgene products is subject to the route of transfection, that is, apical or basal route. Thus, we should cautiously select administration routes in accordance with the purpose. We explain the characteristics of each administration route below.

3.1. Oral route

The oral route is one of the most attractive and challenging routes. Non-invasive administration could be theoretically achieved by the oral route. The potential for daily intake of genetic medicine is also one of the merits of oral administration. Cells in the gastrointestinal tract are transfected via oral routes. Using foreign genes encoding secretion proteins, the transgene products can be secreted into systemic circulation. However, the epithelial barrier, acidic pH in the stomach and digestive fluids are major obstacles for gene transfer via the oral route.

The *in vivo* stability of a recombinant adeno-associated virus (rAAV) type 2 vector could be improved by gastric acid neutralization with sodium bicarbonate and protease inhibition with aprotinin [17]. Despite these changes, the transduction efficiency after oral administration of this vector remained low. We also failed to detect transgene expression after intragastric injection of plasmid DNA in mice [18]. To overcome these obstacles, microparticles and nanoparticles are a promising approach. Chitosan-DNA microparticles could protect the encapsulated plasmid DNA from nuclease degradation [19]. In *in vivo* animal studies, a blue color was observed upon X-gal staining of histological stomach and small intestine sections after oral administration of chitosan-DNA microparticles. Furthermore, chitosan nanoparticles using quaternized chitosan (60% trimethylated chitosan) that were given via a gastric feeding tube exhibited green fluorescent protein expression in the mucosa of the stomach, duodenum, jejunum, ileum and large intestine [20]. Bhavsar and Amiji developed a hybrid system dubbed the nanoparticles-in-microsphere oral system (NiMOS), which consists of gelatin nanoparticles containing plasmid DNA and a poly(epsilon-caprolactone) outer shell [21]. NiMOS resided in the stomach and small intestine for longer than gelatin nanoparticles alone.

In the case of DNA vaccines, transfection into only a subset of antigen-presenting cells may be sufficient for the vaccination to exhibit its required effect. The feasibility of DNA vaccination via the oral route may be high since one or a few administrations is theoretically enough to maintain immunity. In fact, oral DNA vaccines against Mycobacterium tuberculosis using liposome [22] and attenuated Salmonella vector [23] were developed and elicited immune responses.

3.2. Intravenous route

Various targeted gene delivery systems via the intravenous route have been developed worldwide. By intravenous administration, various organs and cells can be targeted. However, undesirable and broad biodistribution of vectors can easily lead to side effects.

Adenoviral vectors have liver tropism after intravenous injection [24]. If the target is not the liver, it is necessary to reduce hepatic transgene expression. Fiber-shaft exchange from adenovirus serotype 5 to serotype 35 in combination with both CAR- and αv integrin-binding ablation by mutation reduced liver tropism [25]. Such mutation may be suitable for retargeting from the liver to other organs/tissues. Capsid engineering of adenoviral fibers from serotype 19p based on phage display technology is useful for targeting to the kidney [26]. On the other hand, when cationic liposome/plasmid DNA complex (lipoplex) was injected intravenously, transgene expression mainly occurred in the lung [27]. Galactosylation of the lipoplex reduced transgene expression in the lung after intravenous injection, while it maintained transgene expression in the liver; however, it remained unselective to the liver [28]. In contrast, we successfully delivered foreign genes to the liver Kupffer cells via the intravenous route by mannosylation of the lipoplex [9].

Innate and adaptive immune responses caused by vector administration are problematic. Recombinant adenoviral vectors induce the production of neutralizing antibodies by single administration [29]. Moreover, neutralizing antibodies to human adenovirus serotype 5 have a prevalence of 60% in Europe [30, 31], 35–70% in North America [32, 33] and 75–100% in Asia [34]; thus, many patients already have neutralizing antibodies before administration of recombinant adenoviral vectors. Neutralizing antibodies also induce complement activation upon administration of recombinant adenoviruses [35]. In addition, an alternative pathway is also activated by recombinant adenoviruses [36]. Neutrophils recognize opsonized adenoviral vectors [37]. These immune responses can cause adverse side effects. In fact, administration of recombinant adenoviral vectors causes liver damage and elevates c-reactive protein in cynomolgus monkey [38]. Moreover, human mortality upon the administration of recombinant adenoviral vectors was reported [39]. On the other hand, non-viral vectors also induce immune responses. Plasmid DNA generally contains an immunostimulatory CpG motif, which is recognized by Toll-like receptor 9 [40, 41]. Lipoplex containing plasmid DNA causes the production of inflammatory cytokines and subsequent liver damage [42, 43]. Immunostimulatory CpG motifs in plasmid DNA also inhibit transgene expression by lipoplex [44]. In addition, dexamethasone treatment was found to improve transgene expression by lipoplex [44]. Here, immunostimulatory CpG motifs can be depleted from plasmid DNA. As expected,

depletion of immunostimulatory CpG motifs from plasmid DNA improves the safety and transgene expression over a long period [45].

When using the intravenous route, it should be considered that interaction with blood components can affect transfection using viral and non-viral vectors. A low level of neutralizing antibodies against adenovirus inhibits CAR-dependent transfection, whereas neutralized adenoviral vector can transfect Fcγ receptor-positive cells [46]. However, this Fcγ receptor-mediated delivery of adenoviral vectors can induce liver inflammation [37, 47]. Binding of coagulation factor X to adenoviral vector serotype 5 determines liver and spleen tropism via heparan sulfate proteoglycan [48-50]. On the other hand, the lipoplex interacts with various blood components due to its cationic nature. Interaction of the lipoplex with serum inhibits *in vitro* transfection, but the inhibitory effect of serum can be overcome by increasing the charge ratio, which is the molar ratio of cationic residues of lipids to anionic residues of DNA [51]. The inhibitory effect of serum on transfection can also be overcome by increasing the lipoplex particle size [52-54]. The lipoplex interacts with complement proteins after intravenous administration in mice; however, the lipofection efficiency and biodistribution of the lipoplex did not change when complement proteins were depleted from mice [55]. Interaction of the lipoplex with plasma lipoproteins decreased transfection efficiency [56, 57]. In contrast, interaction of the lipoplex with erythrocytes greatly inhibited *in vivo* transfection, whereas interaction with serum did not [58, 59]. The lipoplex also induced hemagglutination upon an increase in the charge ratio [60]. Thus, it is necessary to control interaction with blood components for successful and safe *in vivo* transfection using lipoplex. To prevent hemagglutination, coating of cationic carriers with anionic polymers such as γ-polyglutamic acid [61, 62] and chondroitin sulfate [63, 64] is a useful strategy.

Physicochemical properties such as surface charge and particle size of vectors affect *in vivo* transfection, as mentioned above. The size of lipoplex is dependent on the charge ratio and can determine pulmonary transfection efficiency after intravenous injection [65]. In addition, neutral lipids, so-called 'helper lipids', are also important for *in vivo* transfection using lipoplex. While incorporation of DOPE to liposomes is effective in cell culture, incorporation of cholesterol to liposomes enhances pulmonary transfection efficiency [66]. The combination of mannosylated cationic cholesterol derivative with DOPE exhibited superior *in vivo* disposition and transgene expression in the liver than that with DOPC [67]. Incorporation of N-lauroylsarcosine into cationic liposomes in addition to cholesterol inhibited hemagglutination observed in the case of incorporation of DOPE, and increased the pulmonary transfection efficiency [68].

3.3. Local administration

For transfection into a specific organ/tissue/site, local administration is a useful strategy. Local administration can be categorized into the following two routes: vasculature route and non-vasculature route.

Administration routes	Target organs/tissues	Vectors	References
ia	Liver	Naked plasmid DNA	[69]
ia	Pancreas	Adenoviral vector	[70]
ia	Hind limb	Naked plasmid DNA	[71]
ia	Cecum	AAV	[72]
ia	Brain tumor	Adenoviral vector and lipoplex	[73]
ip	Liver	Lipoplex	[28]
riv	Kidney	Naked plasmid DNA	[74]

Abbreviations: ia, intra-arterial; ip, intraportal; riv, retrograde intravenous

Table 1. Administration routes for targeted gene delivery to specific organs/tissues

3.3.1. Vasculature route

Intra-arterial, intraportal and retrograde intravenous routes have been investigated for transfection into a specific target organ. Table 1 summarizes the administration routes and tested target organs.

We developed galactosylated cationic lipoplex targeted to the liver parenchymal cells [8, 28]. Liver-selective transgene expression was observed after intraportal injection of the galactosylated lipoplex, whereas transgene expression was ineffective and non-selective to the liver after intravenous injection [9]. We also developed galactosylated polyethylenimine (PEI)/plasmid DNA complex (polyplex) and analyzed the molecular weight dependence of PEI [75]. For targeted delivery to the liver parenchymal cells, penetration through fenestrated endothelium is one of the major obstacles. We analyzed the intrahepatic disposition characteristics of galactosylated lipoplex [76] and galactosylated PEI polyplex [77]. While galactosylation of carriers was useful to deliver plasmid DNA to the liver, it was proposed that reduction of the particle size of lipoplex would further improve parenchymal cell selectivity by enhancing the penetration through fenestrated endothelium. Here, larger lipoplex exhibited superior transfection efficiency; however, liver parenchymal cell selectivity was low in large lipoplex [78]. In terms of the particle size of lipoplex and polyplex, the composition of the solution is important. Particle sizes of lipoplex and polyplex in non-ionic solution are smaller than those in ionic solution [79, 80]. In the case of siRNA, the particle size of lipoplex is relatively small; using such lipoplexes, several reported studies succeeded in delivering siRNA to hepatocytes *in vivo* [81, 82].

In terms of interaction of the lipoplex with serum, we reported that transgene expression in the liver after intraportal injection of galactosylated lipoplex was increased by pre-incubation of the lipoplex with serum [83]. This enhancement of transgene expression in the liver was also observed in conventional lipoplex [84]. Multiple components in serum including calcium ion, aggregation-inhibiting components, fibronectin and complement component C3 were responsible for increased transgene expression in the liver [84].

Target organs/tissues	Vectors	References
Skeletal muscle	Naked plasmid DNA	[85]
Heart	Naked plasmid DNA	[86]
Heart	AAV	[87]
Liver	Naked plasmid DNA	[88]
Kidney	Lentiviral vector	[89]
Spleen	Naked plasmid DNA	[90]
Stomach	Naked plasmid DNA	[91]
Thymus	Adenoviral vector and others	[92]
Tumor	Naked plasmid DNA	[93]
Tumor	Naked plasmid DNA and lipoplex	[94, 95]

Table 2. Direct injection for targeted gene delivery to specific organs/tissues

3.3.2. Non-vasculature route

Direct injection to the target organ such as the liver or spleen has been investigated (Table 2). By direct injection to the target organ, the use of naked plasmid DNA without carrier systems is sufficient to detect transgene expression. However, in general, transgene expression is limited to the injection site. To overcome a limited transfection area, electroporation after intramuscular injection of plasmid DNA increased the number of transfected myofibers [96].

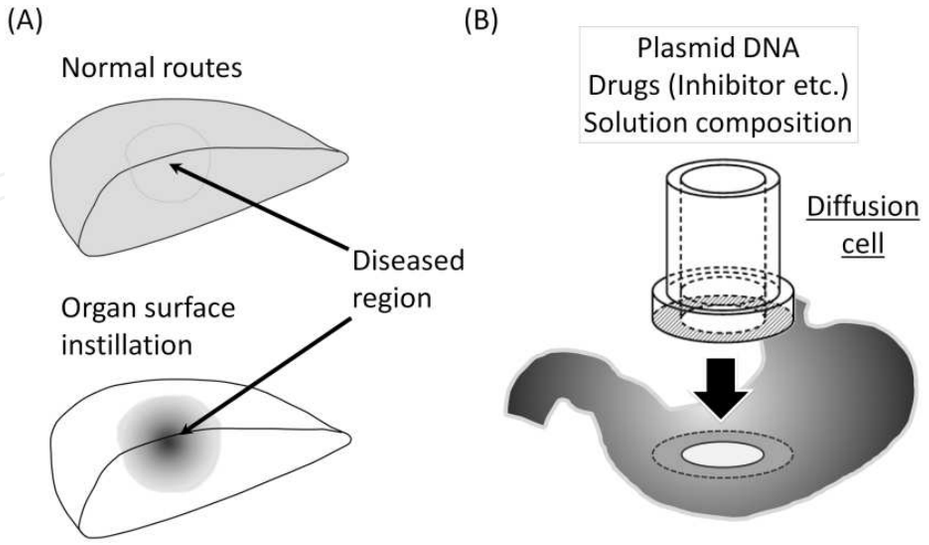

Figure 2. Scheme of organ surface instillation. Panel (A) represents the proposed drug distribution after systemic administration and organ surface instillation of drugs. Panel (B) represents attachment of a glass-made cylindrical diffusion cell onto the organ surface.

For other routes of gene transfer, retrograde intrabiliary injection of naked plasmid DNA, polyethylenimine-plasmid DNA complex and chitosan-plasmid DNA complex resulted in transgene expression in the liver [97]. Intranasal administrations of adenoviral vector [98], lipoplex and polyplex [99] were also tested. In addition, inhalation of chitosan/plasmid DNA nanoparticles resulted in pulmonary transgene expression [100]. Intracerebroventricular administration of lentiviral vector was utilized to deliver foreign genes to the brain [101]. Gene gun bombardment of plasmid DNA with gold particles resulted in efficient gene transfer to the skin [102]. After intraperitoneal injection of adenoviral vector, not only mesothelium but also parenchymal cells of the liver were transduced [103]. This non-specific biodistribution was overcome by ablation of native CAR and integrin receptor binding [103].

3.4. Organ surface route

We developed a novel route for targeted gene delivery to intra-abdominal and intra-thoracic organs, namely, the organ surface route (Fig. 2A). When diseases are limited to a certain region, the organ surface route enables us to target the diseased region, while drugs are distributed to the whole organ via the normal route. Naked plasmid DNA was utilized to transfect target organs/sites. As a first report of this approach, the liver was targeted and successfully transfected in mice [104]. Selectivity of transgene expression in the applied liver lobe was high. Laparotomy was performed in the first reported study, but it is not essential since catheter-based administration through the abdominal wall is available [105]. This catheter-based administration is essential to the safety of liver surface instillation of plasmid DNA [106].

We developed an experimental system using a glass-made cylindrical diffusion cell attached to the organ surface (Fig. 2B) [107]. Using this experimental system, we can precisely limit the area of drug application. Specific transgene expression in the applied area of the liver was achieved [108]. The effect of solution composition on naked plasmid DNA transfer was also examined [109]. Use of hypotonic solution enhanced the transfection efficiency in the applied site of the liver. As for the mechanism of transfection, we analyzed endocytic routes for naked plasmid DNA transfer *in vivo*. While the lipoplex and polyplex are taken up via clathrin- and caveolae-mediated endocytosis [110-113], macropinocytosis is essential for naked plasmid DNA uptake in mesothelial cells in mice [114].

As for other organs, unilateral kidney [115], unilateral lung [116], spleen [117] and stomach surface [118, 119] were transfected with naked plasmid DNA in mice. To improve organ selectivity, microinstillation of naked plasmid DNA onto the stomach was performed [18]. Since specific transgene expression in the stomach was observed in rats [120], organ size would be an important factor for target selectivity of gene transfer. Moreover, specific transgene expression in the applied liver lobe was also achieved in mice by controlling instillation speed using an infusion pump [121].

3.5. Comparison of administration routes

We summarize the advantages and disadvantages of each administration route for targeted gene delivery in Table 3.

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Administration routes	Advantages	Disadvantages
Oral	Ease of administration, Frequent dosing (daily intake)	Barriers (epithelium, digestive fluids), Low selectivity
Intravenous	Frequent dosing, Vast distribution	Non-specificity
Intra-arterial, Intraportal, Retrograde intravenous	Selective delivery	Necessity of cannulation
Direct injection	Effective gene transfer, High selectivity	Physical force against the organ, Limited region, Limited frequency of dosing
Intraperitoneal	Effective gene transfer	Low selectivity
Organ surface	Effective gene transfer, High selectivity	Necessity of laparoscopy

Table 3. Advantages and disadvantages of vector transfer routes.

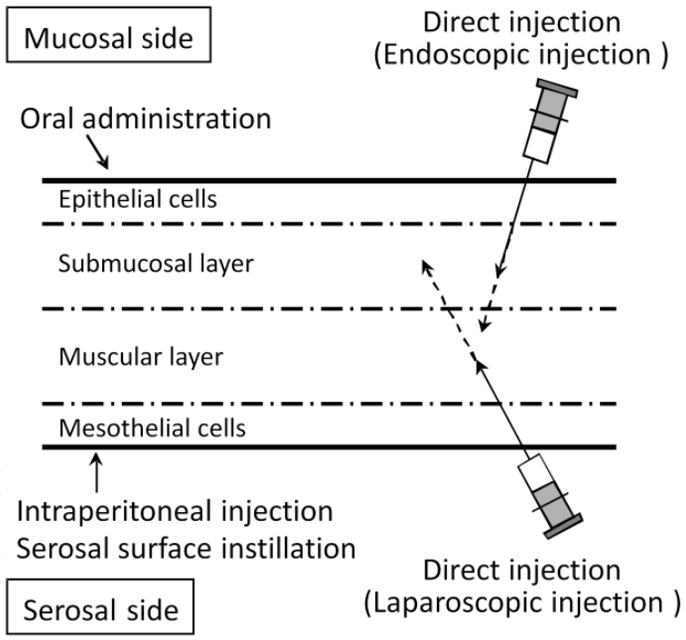

Figure 3. Scheme of administration routes for targeted delivery of foreign genes to the stomach

Direct injection of rAAV vector to the liver exhibited faster and stronger transgene expression than intravenous and intraportal injections of rAAV vector [122]. Similar results were obtained for direct injection of the lipoplex into localized intrahepatic tumors [123]. Moreover, direct intrahepatic injection of adenoviral vector reduced inflammation and increased transgene expression in comparison with intravenous injection [124]. On the other hand, ret-

rograde infusion of lentiviral vector into the ureter, injection into the renal vein or artery, and direct injection into the renal parenchyma were compared [89]. Parenchymal or ureteral administration appeared to be more efficient than other routes of administration.

Figure 3 depicts the administration routes for targeted gene delivery to the stomach. Via the oral route, there are many barriers such as digestive fluids and acidic pH that hamper effective gene transfer. Although effective gene transfer can be achieved by direct injection, it is necessary to consider tissue damage. In contrast, safe and effective gene transfer is possible by serosal surface instillation of naked plasmid DNA. Although transgene expression is limited to the surface layer in the case of serosal surface instillation, limited vertical distribution of transgene products can be overcome by the use of the secretory form of proteins [121].

4. Improving methods for targeted gene delivery

Various strategies have been tested to improve targeted gene delivery. Methods for improved targeted gene delivery can be categorized as physical approaches and chemical approaches.

Physical forces such as electroporation, sonoporation and mechanical massage have been employed to improve targeted gene delivery. Naked plasmid DNA can be delivered to the liver by intravenous injection with electroporation [125, 126]. Intravenous injection of naked plasmid DNA with tissue electroporation resulted in significant transgene expression in the liver, spleen and kidney, but not in the skin or muscle [127].

Utilization of microbubbles with ultrasound exposure can deliver naked plasmid DNA to the muscle [128, 129], liver [130] and lung [131]. Use of PEGylated liposomal bubbles containing perfluoropropane with ultrasound exposure was also effective to deliver naked plasmid DNA via the femoral artery [132]. Mannosylated lipoplex and liposomal bubbles with ultrasound exposure can transfect the liver and spleen [133]. In addition, mannosylated PEGylated bubble lipoplexes selectively transfected antigen-presenting cells *in vivo* [134]. DNA vaccination by this type of lipoplex with ultrasound exposure resulted in suppression of melanoma growth and metastasis [135]. The timing of ultrasound exposure was important [136]. As a mechanism of high transgene expression, a transcriptional process activated by ultrasound exposure was involved [137].

Hydrodynamics-based transfection, with rapid large volume injection of naked plasmid DNA via the intravascular route, is an efficient method to transfect the liver [138, 139]. It was also reported that pig liver can be transfected by retrograde hydrodynamic injection of plasmid DNA via an isolated segment of the inferior vena cava [140]. In terms of the mechanism of high efficiency of gene transfer in hydrodynamics-based transfection, both the generation of transient pores [141, 142] and a transcriptional process activated by hydrodynamic injection [143, 144] are important.

Naked plasmid DNA was also intravenously delivered to the liver by mechanical massage of the liver [145]. Pressure-mediated deliveries of naked plasmid DNA to the kidney [146], liver

and spleen [147] were also achieved. As the mechanism of high transgene expression, a transcriptional process activated by pressure to the tissue was involved [148].

Chemical modification of gene carriers has also been investigated. PEGylation of carriers improves blood circulation of the carrier and tumor accumulation by the enhanced permeability and retention effects [149]. However, transfection efficiencies of PEGylated vectors are generally low. Although PEGylation of lipoplex reduced retention in the lung and heart, PEGylated lipoplex failed to deliver foreign gene into tumors [150]. PEGylation of adenoviral vectors generally prevents CAR recognition. Hexon-specific PEGylation of adenoviral vector improved *in vitro* transfection efficiency in the presence of neutralizing antibodies, *in vivo* blood retention and tumor accumulation after intravenous administration; however, transfection efficiency in tumor remained low [151]. To overcome this dilemma of PEGylation, that is, high retention and low uptake, cleavable PEG-lipids have been developed. PEG-lipids, which were designed to exhibit cleavage of the PEG moiety by tumor-specific matrix metalloproteinase, were incorporated into a multifunctional envelope-type nano-device [152]. As a result, transgene expression in the tumor was stimulated after intravenous injection of this carrier in comparison with that with normal PEGylated gene carrier.

It was reported that incorporation of human serum albumin to lipoplex enhanced the transfection efficiency *in vitro* and *in vivo* [153]. Moreover, utilization of serum components such as asialofetuin [154], transferrin [155] and fibronectin [156] was tested for the development of vectors.

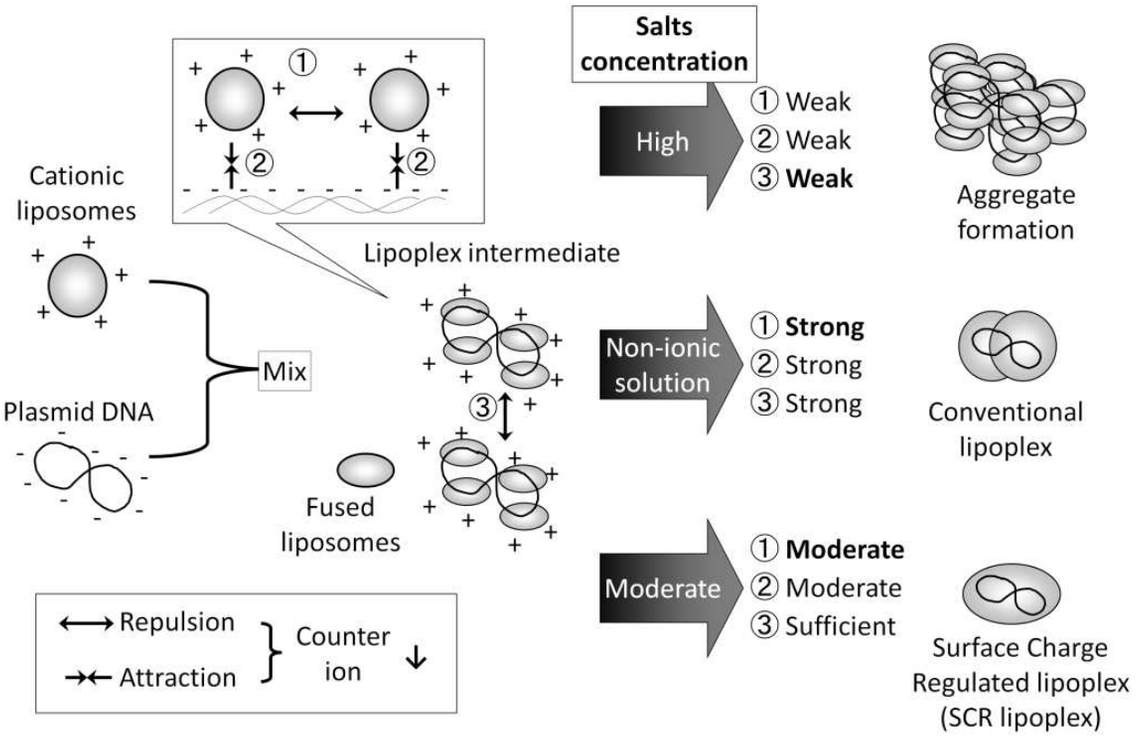

Figure 4. Schematic representation of surface charge-regulated lipoplex.

Intravenous sequential injection of cationic liposome and plasmid DNA resulted in significant pulmonary transgene expression with reduced inflammatory cytokine production compared with those with the lipoplex [157]. Sequential injection resulted in lower DNA uptake by the liver and higher DNA levels in the lung than with the lipoplex administration [158]. Interaction with several serum proteins including albumin reduced inflammatory cytokine production by sequential complex (liposome mixed with serum proteins before mixing with plasmid DNA), whereas interaction of the lipoplex with serum proteins did not reduce inflammatory cytokine production by lipoplex [159].

We successfully developed surface charge-regulated (SCR) lipoplex, which improved targeted gene delivery by stabilizing the lipoplex. Figure 4 shows a scheme of the salt-dependent formation of lipoplex. For *in vivo* preparation of the lipoplex, the concentrations of plasmid DNA and liposomes are high; consequently, a physiological concentration of salts induces aggregation of the lipoplex. This problem can be overcome using a non-ionic solution such as 5% glucose solution. Here, we hypothesized that repulsion between cationic liposomes was too strong to induce sufficient fusion of lipid membranes for stable lipoplex formation. Moderate concentration of salts in the solution of the lipoplex would reduce repulsion among cationic liposomes and enhance fusion of lipid membranes, while maintaining sufficient repulsion among lipoplex particles. This hypothesis was proved by a series of physicochemical experiments including fluorescent resonance energy transfer assessments and measurements of particle size changes in the presence of physiological concentration of salts [160]. This stable galactosylated SCR lipoplex exhibited superior hepatocyte-selective gene transfer than conventional lipoplex after intraportal injection [160]. Furthermore, the stabilization effect of SCR lipoplex was also evident in pulmonary gene transfer after intravenous injection [161].

As for the organ surface instillation method, we succeeded in enhancing the transfection efficiency of naked plasmid DNA by several strategies. Pretreatment with epidermal growth factor (EGF) enhanced transgene expression and increased transgene-positive cells on the stomach after instillation of naked plasmid DNA onto it [162]. Rubbing the gastric serosal surface with a medical spoon after instillation of naked plasmid DNA onto the stomach was more effective than EGF pretreatment [163]. However, rubbing the organ surface with a medical spoon may be impractical for future clinical application. Thus, we searched for various materials to reproduce the effect of rubbing an organ's surface. Among them, concomitant use of calcium carbonate suspension with naked plasmid DNA was similarly effective as rubbing the gastric serosal surface [164]. Unfortunately, sedimentation of calcium carbonate suspension occurs rapidly and is problematic. To obtain slowly settling particles of calcium carbonate, we tested various conditions for calcium carbonate synthesis. We succeeded in synthesizing a novel form of calcium carbonate with a flower-like shape, named calcium carbonate microflowers [164]. Sedimentation of calcium carbonate microflowers was sufficiently slow to perform *in vivo* experiments. Fortunately, the suspension of calcium carbonate microflowers containing naked plasmid DNA was a more effective transfection reagent than commercially available calcium carbonate, especially at a low concentration of calcium carbonate. Intraperitoneal injection of the suspension of calcium carbonate microflowers containing naked

plasmid DNA resulted in effective and peritoneal cavity-selective gene transfer. However, the mechanism of effective *in vivo* transfection remains to be elucidated.

5. Disease-dependent strategies in targeted gene delivery

Among the above-mentioned methods, intramuscular injection of naked plasmid is one of the simplest methods since it can be applied without surgery and carriers. Not only muscular diseases, such as dystrophy, but also systemic diseases may be cured using the secretory form of proteins. Intramuscular injection of plasmid DNA encoding hepatocyte growth factor rescued critical limb ischemia with high safety in a phase I/IIa clinical trial [165]. Muscular delivery of naked plasmid DNA encoding erythropoietin resulted in an increase of hematocrits [166]. In general, however, targeted gene delivery to specific organs/sites/cells is required since high biological activities of proteins may lead to side effects. For example, in suicide gene therapy to treat tumors, thymidine kinase gene expression should be restricted to tumor cells [167]. Since hepatocyte growth factor is mitogenic, liver-directed gene transfer is a rational approach to treat liver cirrhosis [168]. To treat inherited gene deficiency diseases such as familial hypercholesterolemia (LDL receptor deficiency in hepatocytes) [169] and Crigler-Najjar syndrome (uridine diphospho-glucuronosyl transferase 1A1 deficiency in hepatocytes) [170], targeted gene delivery is also reasonable due to its efficacy.

As for DNA vaccination, Kasinrerk et al. compared intramuscular, intraperitoneal, intravenous and intrasplenic immunizations with a single dose of naked plasmid DNA and observed that only the intrasplenic route induced specific antibody production [171]. In contrast, to develop DNA vaccine to induce cellular immunity, intradermal injection of naked plasmid DNA with electroporation was better than intrasplenic injection, even though there was high transfection efficiency in the spleen [172]. Gene gun bombardments of naked plasmid DNA to the skin were not effective to induce cellular immunity in comparison with intracutaneous injections of antigen-transduced dendritic cells [102]. Gene gun bombardments of naked plasmid DNA to the skin induced Th2 response and anaphylactic shock upon antigen recall [173]. On the other hand, transgene expression of fusion proteins of the immunodominant domain of human type XVII collagen and dendritic cell-specific antibody targeted to dendritic cells in the skin induced tolerance to human type XVII collagen in a skin transplantation model [174]. Intraperitoneal injection of mannosylated lipoplex resulted in efficient transgene expression in antigen-presenting cells and induced cellular immunity [175, 176]. As for the intravenous route, mannosylated lipoplex initiated a Th1 response [177]. As mentioned above, mannosylated PEGylated bubble lipoplexes with ultrasound exposure more effectively and selectively transfected antigen-presenting cells than an approach without ultrasound exposure after intravenous injection, and induced strong cellular immunity [134, 135]. Thus, the success or failure of DNA vaccination is dependent on transfection methods including transfection routes.

6. Conclusions

Selection of administration routes is important in targeted gene delivery for not only efficacy but also safety of the vector. Administration routes can be categorized as systemic routes and local routes. Via the systemic routes, administration is simple and does not require a complicated operation. However, very wide distribution of the vectors after their systemic administration may lead to systemic side effects. This problem can be overcome by changing the administration route from a systemic route to a local route. In addition, target selectivity can be improved by modification of the vectors with a ligand, combination with targeted application of physical forces and utilization of tissue-specific promoters. Importantly, selection of administration routes can be combined with these strategies to improve targeted gene delivery. The importance of selection of administration routes is dependent on the kind of target disease. Taking safety including germline conservativeness into consideration, further improvement of targeted gene delivery systems should be pursued.

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