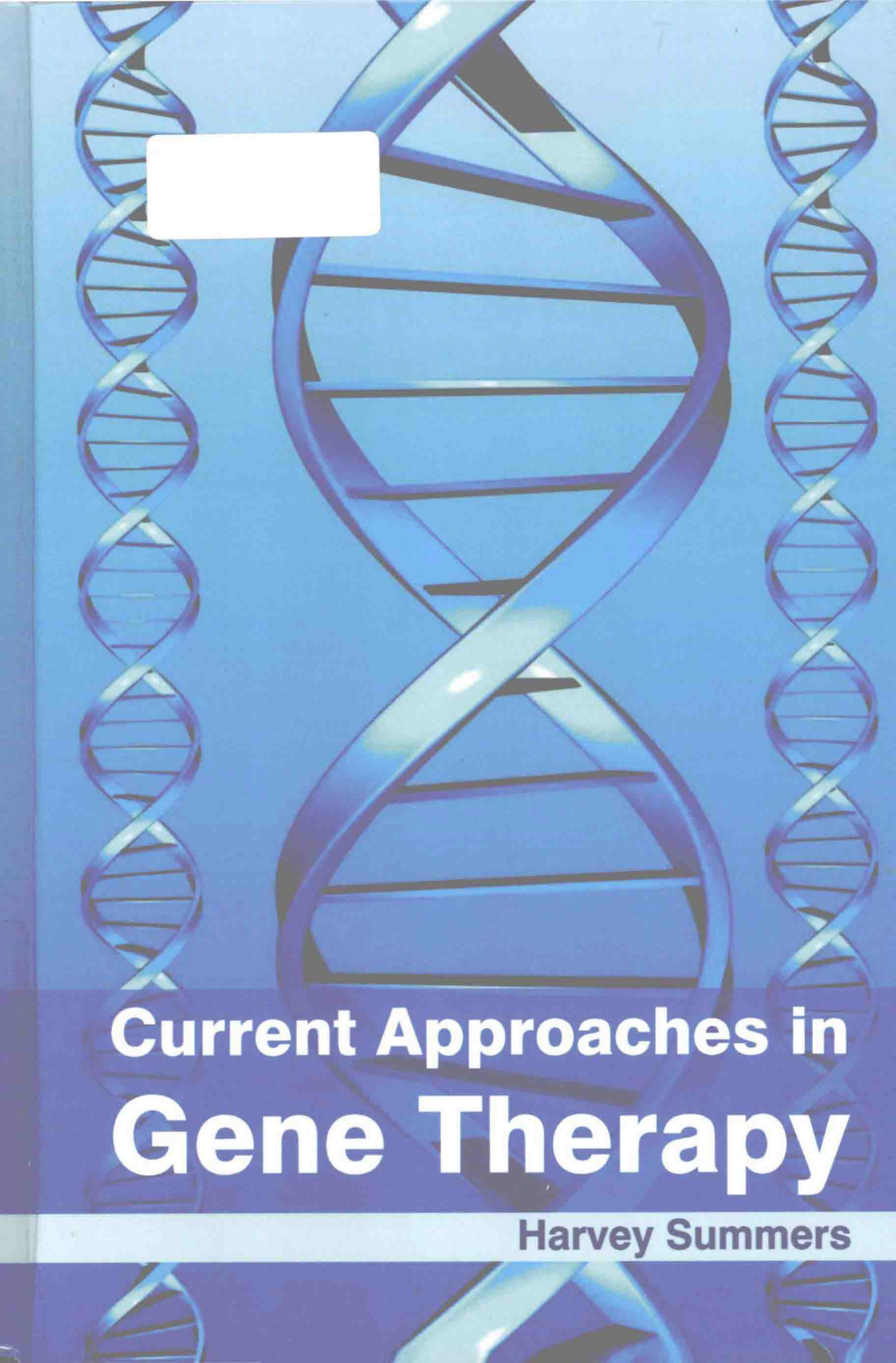
Current Approaches in
Gene Therapy
Harvey Summers

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Edited by **Harvey Summers**

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Current Approaches in Gene Therapy

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Preface

This book intends to present updated reviews of the swiftly rising field of gene therapy. Gene therapy has the capability to be a suitable therapeutic with enhanced specificity and reduced side effects that can offer a cure for several disorders. The book encompasses a broad spectrum of topics including methodologies of gene delivery and recognition of targets in cancer gene therapy. The ultimate aim is to achieve the complete benefit of such an innovative approach for the treatment of several diseases, including cancer, by educating the readers regarding the true nature and potential of gene therapy.

This book is the end result of constructive efforts and intensive research done by experts in this field. The aim of this book is to enlighten the readers with recent information in this area of research. The information provided in this profound book would serve as a valuable reference to students and researchers in this field.

At the end, I would like to thank all the authors for devoting their precious time and providing their valuable contribution to this book. I would also like to express my gratitude to my fellow colleagues who encouraged me throughout the process.

Editor

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Section 1

Approaches to Gene Therapy

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Chapter 1

Targeted Gene Delivery: Importance of Administration Routes

Shintaro Fumoto, Shigeru Kawakami,
Mitsuru Hashida and Koyo Nishida

Additional information is available at the end of the chapter

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].

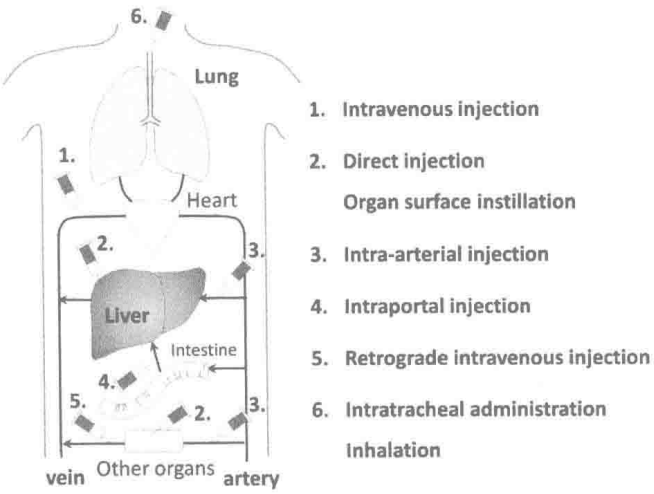

Figure 1. Scheme of administration routes for targeted gene delivery.

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].

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