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Review

Theme: Emerging Drug Delivery Technologies
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Physical Approaches for Nucleic Acid Delivery to Liver

Kenya Kamimura[1] and Dexi Liu[1,2]

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Abstract. The liver is a key organ for numerous metabolic pathways and involves many inherited diseases that, although being different in their pathology, are often caused by lack or overproduction of a critical gene product in the diseased cells. In principle, a straightforward method to fix such problem is to introduce into these cells with a gene-coding sequence to provide the missing gene product or with the nucleic acid sequence to inhibit production of the excessive gene product. Practically, however, success of nucleic acid-based pharmaceutics is dependent on the availability of a method capable of delivering nucleic acid sequence in the form of DNA or RNA to liver cells. In this review, we will summarize the progress toward the development of physical methods for nucleic acid delivery to the liver. Emphasis is placed on the mechanism of action, pros, and cons of each method developed so far. We hope the information provided will encourage new endeavor to improve the current methodologies or develop new strategies that will lead to safe and effective delivery of nucleic acids to the liver.

KEY WORDS: gene delivery; liver; nonviral vectors; physical method; transfection.

INTRODUCTION

The liver is the largest organ in the body with functions crucial to sustaining life and is responsible for many genetic and metabolic disorders. Significant efforts have been made in the past toward elucidation of mechanisms underlying different liver diseases. Progress made in both basic and clinical research in recent years has led to the development of gene therapy as an alternative to orthotropic liver transplantation, which is the only effective therapy currently available for many liver diseases. The challenge for liver gene therapy, however, is the development of a method to allow safe and effective delivery of therapeutic gene to liver cells. The following sections summarize the fundamental aspects of hepatic gene delivery with the emphasis on physical approaches.

STRUCTURE AND PHYSIOLOGY OF THE LIVER AND BARRIERS OF NUCLEIC ACID DELIVERY

The liver is situated just below the diaphragm and the upper right side of the stomach. In human, it comprises about 2% of the total adult body weight and consists of four lobes (right, left, quadrate, and caudate lobe). At the microscopic and functional level, the liver is composed of lobules each of which ranges from 1 to 2.5 mm in diameter and contains a mass of cells (2×10^5 cells per milligram) (1). The outline of the lobules in human liver are irregular, but in some of the lower animal species (for example, the pig), they are well-defined and have hexagonal shape (2). The base of the lobule is clustered around the smallest hepatic vein (central vein). The remaining part of each lobule is imperfectly isolated from the surrounding lobules by a thin stratum of connective tissue in which a plexus of blood vessels and ducts is contained (1,3). In some animals, as in the pig, the lobules are completely isolated from one another by the interlobular connective tissue (2).

The liver consists of plural types of cells. The hepatocytes are in polyhedral shape. They vary in size from 12 to 25 μm in diameter and contain one or sometimes two distinct nuclei in each cell. The hepatocytes face, the perisinusoidal space called space of Disse. The neighboring hepatocytes are connected by tight junctions, gap junctions, and desmosomes. The sinusoids are made of endothelial cells, phagocytic Kupffer cells, stellate cells (Ito cells), and pit cells. The Kupffer cell is the macrophage attached to the sinusoidal endothelium and responsible for the removal of invading particles into the blood. Ito cells lie in the space of Disse and have a function of storage of retinoids, and with hepatic injury, they transform to myofibroblast-like cells and produce fibrous tissue. Pit cells are one of the natural killer cells types that are attached to the sinusoidal surface of the endothelium (1).

The liver has an unusual blood supply system. Approximately 1,300 mL of blood flow into the liver every minute, representing about 25% of total cardiac output. About 80% of the liver blood is transported *via* the portal vein carrying nutrients or digested food from the digestive tract. The other 20% come *via* hepatic artery carrying oxygen-enriched blood from the heart. The hepatic artery and the portal vein branch into a network of small blood vessels that empty into the sinusoids where the venous and arterial blood mix (1). The endothelial wall of the sinusoids is discontinuous (or fenestrated) with pores of about 100 nm in diameter, which brings

[1] Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, 527 Salk Hall, Pittsburgh, PA 15261, USA.
[2] To whom correspondence should be addressed. (e-mail: dliu@pitt.edu)

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blood substances or particles below 100 nm into direct contact with the liver cells beyond the endothelium. The sinusoids drain into the central veins which join to form the hepatic vein, from which blood leaves the liver, enters the inferior vena cava, and returns to the heart (1).

The bile ducts begin at little passages in the liver cells that communicate with bile capillaries. These passages are merely little channels or spaces left between the contiguous surfaces of two or more hepatocytes. These channel-like bile ducts are always separated from the blood capillaries by at least half the width of a liver cell and open into the interlobular bile ducts which run in Glisson's capsule accompanying with the portal vein and hepatic artery. The walls of the bile ducts consist of a connective tissue coat in which there are muscle cells arranged both circularly and longitudinally and an epithelial layer consisting of short columnar cells resting on a distinct basement membrane. The exterior coats of the large bile ducts is composed of strong fibrotic tissue with a certain amount of muscular tissue arranged for the most part in a circular manner around the duct. The interior mucous coat of the bile ducts is continuous with the lining membrane of the bile ducts and gallbladder and also with that of the duodenum. The bile juice enters the duodenum through papilla (1).

The functions of the liver are numerous, working closely with nearly every system and process in the human body. The hepatic parenchymal cells have a broad range of synthetic and catabolic functions. The liver is the primary organ responsible for the metabolism of carbohydrates, lipids, proteins, and heme and for removal of toxins, hormones, and aged red blood cells. The liver is responsible for synthesizing most plasma proteins (with the exception of immunoglobulins), bile acids, cholesterol, and heparin and serves as the principal site for storage of iron, glycogen, lipids, and vitamins. The liver also plays an important role in the detoxification of many drugs and excretion of metabolic end products such as bilirubin, ammonia, and urea.

Because of its sophisticated and important function in regulating metabolism and maintaining homeostasis, the liver is a key organ for most metabolic pathways, and therefore, numerous inherited diseases have their origin in this organ. Candidate diseases include genetic disorders such as hemochromatosis, hemophilia A and B, alpha 1 antitrypsin deficiency, Wilson's disease, Crigler–Najjar syndrome type I, ornithine transcarbamylase deficiency, type IIa familial hypercholesterolemia, and afibrinogenemia. Therefore, the medical significance and afferent and efferent pathways to the liver have made the liver an ideal target for gene therapy studies.

The primary barrier for nucleic acid delivery to liver cells is the plasma membrane. If DNA molecules are larger than 100 nm, the endothelium also serves as the barrier for intra-hepatocyte delivery. As far as gene delivery to the liver is concerned, the true challenge is to deliver nucleic acids to most hepatocytes in the liver, if not all, without causing tissue damage.

CONSIDERATIONS ON NUCLEIC ACID DELIVERY FOR TREATMENT OF LIVER DISEASES

The most crucial aspect of nucleic acid-based therapy for liver-associated diseases is the availability of a suitable delivery system. The subject of gene delivery and gene therapy has been well studied in the past 30 years primarily with viral vectors (4,5). Analogous to liver transplantation, early work on liver gene therapy employed *ex vivo* strategy involving re-implantation of the patient's hepatocytes after viral gene transfer. In fact, this strategy was used for liver-directed gene therapy against hypercholesterolemia (6). Due to the invasiveness and high cost of the *ex vivo* approach, much efforts have been made to find an alternative and easier *in vivo* method. To target the liver specifically, researchers have opted to perform injections of viral vectors directly into the afferent vessels of the liver (portal vein) or the bile duct instead of the peripheral circulation. However, viral vectors employed in these studies entail some disadvantages. For example, retroviral vector-mediated delivery necessitates partial hepatectomy to trigger hepatocyte division (7). Adenoviral vectors induce immune response that causes the destruction of transduced hepatocytes (8) and prevents repeated administration. Adeno-associated viral vector with small loading capacity suffers from the same problem of immune response. While immune suppression of the host improved transgene expression and decreased liver destruction (9), the necessity of continued suppression to maintain the level of gene expression renders this approach less ideal for human gene therapies.

Another approach for liver-specific gene delivery is the use of synthetic compounds called synthetic vectors. Much work for gene delivery to the liver using synthetic vectors has focused on targeted gene delivery through asialoglycoprotein receptor (ASGP-R) (10) and subsequently the transferrin receptor (11). ASGP-R is a membrane protein localized to the sinusoidal surface of hepatocytes. It is a prototypic member of the C-type lectin family in mammals. Its ligand-binding affinity is dictated by the number (3>2>1), type (GalNAc>Gal), and conformation of terminal residues on N-glycans. A natural triantennary N-glycan possesses relatively high affinity (K_d=4 nM) due to a precise geometric fit between three terminal galactose residues and complementary binding pockets on the ASGP-R (10,12). The receptor can recognize a variety of other galactosylated polymers, many of which have been used in gene delivery. The ASGP-R internalizes its ligand *via* coated pits, de-associates from ligand in a prelysosomal compartment, and then recycles to the cell surface (10). Early studies by Wu and colleagues (13) and a more recent study by our laboratory (14) demonstrated *in vivo* gene delivery by targeting the ASGP-R following intravenous injection of plasmid DNA/carrier complex. The work by Kwoh and coworkers (15), Hashida and coworkers (16), and Rice and coworkers (17) have also demonstrated the utility of ASGP-R targeted gene delivery as a model system for studying the relationship between carrier structures and transient gene expression. Studies from Kwoh *et al.* and Rice's group indicate that specific targeting of electropositive DNA complexes to hepatocytes requires the use of targeting ligand and a large volume of solution for injection (15,17). Unfortunately, the gene expression achieved in each of these studies has been low and short in duration.

Concerns on virus-induced toxicity and low delivery efficiency of synthetic vectors including those of cationic lipids (18), cationic polymers (19), and naturally occurring

compounds (20) have inspired significant effort in recent years to find new method for nucleic acid delivery in its original or “naked” form. An obvious advantage of naked DNA is its simplicity and safety. As part of composition in biological system, nucleic acids as a chemical identity are not toxic, readily biodegradable, and impermeable across the cell membrane. The following sections will summarize the progress in the area of naked DNA delivery. Although discussion was centered on gene delivery, the techniques and the underlying principles are applicable to other forms of nucleic acids such as DNA, RNA, and nucleic acid-based derivatives. The overall emphasis of our discussion is on the rationale, pros, and cons of each method for liver-based nucleic acid delivery.

PROGRESS IN DEVELOPMENT OF PHYSICAL METHODS FOR NUCLEIC ACID DELIVERY TO LIVER

At the molecular level, the objective of delivery of nucleic acid sequence to the liver is to accomplish one of the following: (a) replacement of a missing gene product; (b) overproduction of a gene product; (c) DNA vaccine; (d) generation of hormonal proteins to regulate cell growth (e.g., growth hormone), cell differentiation (e.g., cytokines), or metabolism (e.g., insulin); (e) inhibition of gene expression (delivery of oligonucleotides, siRNA, shRNA); or (f) DNA repair (single-strand DNA or DNA/RNA hybrid). Because nucleic acids are nonpermeable across cell membrane due to their large size and hydrophilicity, the physical method of intrahepatic nucleic acid delivery aims at overcoming the plasma membrane barrier of liver cells. The routes of delivery could be direct injection into the liver, intraportal or intrahepatic vein, intrahepatic artery, intrabile duct, or systemic. The physical forces employed include pressure, shock wave, electric pulse, and ultrasound wave. Table I summarizes various aspects of currently available physical methods for nucleic acid delivery to the liver.

Intrahepatic Gene Delivery by Needle Injection

Inspired by the success of gene delivery to muscle cells through direct injection of plasmid DNA into muscle in mice (21), Hickman *et al*. injected reporter plasmids into mouse liver and demonstrated gene expression in liver cells, primarily near the needle track (22). A similar procedure was also performed as the treatment of hepatocellular carcinoma involving intratumor injection of plasmid DNA containing *p53* gene (23). Although three of the five patients who received percutaneous injection of wild-type p53 showed objective tumor response with reduction of the tumor size and decreasing of serum alpha-fetoprotein, gene delivery was limited to cells on and near the needle track and the overall delivery efficiency was extremely low. The mechanism of intracellular gene delivery by needle injection appears to involve the penetration of sharp needle across the cells on its path, breaking cell membrane and allowing plasmid DNA to enter the cells before the broken cell membrane reseals. Consequently, tissue damage is always produced with this procedure and the total number of cells with successful nucleic acid delivery is limited.

Intrahepatic Gene Delivery by Gene Gun (Ballistic Bombardment)

Gene gun-based gene delivery was first employed in 1987 for gene delivery to plant cells (24), and since the 1990s, has been used in cultured cells and *in vivo* for transfection (25). It involves propelling the DNA-coated gold particles against cells. Intracellular gene transfer is achieved by adjusting the propelling pressure, particle size, and distance between the gene gun and target cells. Yang *et al*. (26) and Kuriyama *et al*. (27) have shown in mouse that gene transfer to liver cells can be achieved using the gene gun approach. However, the procedure requires a surgical procedure to expose the liver and gene expression was found only in cells near the exterior surface of the targeted area. Because of the shallow penetration of DNA, particle bombardment through a gene gun appears more appropriate for gene delivery to skin for vaccination and immune therapy (28–32). Roberts *et al*. have demonstrated the safe and effective particle-mediated epidermal delivery of DNA vaccine against hepatitis B virus (HBV) (30). Chang *et al*. have reported enhancement in efficiency by repeated bombardment (33).

Intrahepatic Gene Delivery by Electroporation

Electroporation was first utilized for gene transfer to mammalian cells by Neumann *et al*. in 1982 (34) and has been extensively studied in recent years as an effective method for gene delivery not only *in vitro* but also *in vivo* (35). As evidenced by its name, gene transfer by this technique is achieved by generating membrane pores on cells through electric pulse, normally requiring a high voltage applied to a relatively small area of tissue. The delivery efficiency is determined by the pulse intensity, duration and frequency,

Table I. Features of Physical Approach for the Delivery of Nucleic Acid to the Liver

Method	Force	Application	Advantages	Limitation/problem
Needle injection	Mechanic	Intratissue	Simple	Low efficiency, limited to needle track
Gene gun	Momentum	Topical	Good efficiency	Limited to small area, need surgical procedure for internal organs
Electroporation	Electric pulse	Topical, intratissue	High efficiency	Limited to small area, need surgical procedure, tissue damage
Sonoporation	Shock wave	Intratissue	Region specific	Low efficiency, tissue damage
Hydrodynamic delivery	Hydrodynamic pressure	Intravascular	Simplicity, high efficiency, region-specific	Need catheterization in large animals

and the type of cells as well (36). Using this method, Heller *et al.* (37) and Suzuki *et al.* (38) reported the successful naked DNA transfer to the liver in rats in the late 1990s. Liu *et al.* reported efficient gene transfer to mouse liver by electroporation following tail vein administration of the naked DNA (39). Sakai *et al.* showed that electric pulses applied to the mouse left lateral lobe after intravenous injection of naked plasmid DNA results in regional gene expression centered on the area where the pulses were applied (40). Transfected cells were more broadly distributed with the systemic injection compared to a local injection. These reports suggest that systemic injection is preferable to the regional injection of DNA to the liver when electroporation was used. By employing this procedure, Jaichandran *et al.* demonstrated the phenotypic correction in hemophilic mice when plasmid containing cDNA of factor VIII was transferred into the mouse liver (41). The major drawback of the electroporation-mediated gene transfer to liver cells *in vivo* is the involvement of surgical procedure to expose and allow insertion of the electrodes into the liver or placement of the plate electrodes onto the liver surface. In addition, the area impacted by each procedure is rather limited. While effective in increasing the number of transfected cells, high voltage often results in significant tissue damage. It appears that a new device with optimal electrical parameters and specifically designed electrodes is needed before electroporation can be clinically useful for liver gene transfer.

Ultrasound-Mediated Gene Transfer

This technique was developed in the 1990s to facilitate gene transfer to mammalian cells *in vitro* (42–44) and later in tissue (45–63). A more popular name reflecting the mechanism of action for this technique is sonoporation, indicating the creation of acoustic membrane pores on the cells through which nucleic acids diffuse into cells. Gene transfer efficiency appears to be controlled by pulse intensity, frequency, and duration (64). Sonoporation *in vitro* often results in high cell mortality with a small fraction of surviving cells showing gene transfer. The potential for *in vivo* use has been a subject of intensive research. Sonoporation-enhanced gene transfer has been explored in the cornea (46), brain (47), central nervous system (48,49), spinal cord (50), bone (51), peritoneal cavity (52), kidney (53), pancreas (54), liver (55,56), embryonic tissue (57), dental pulp (58), solid tumor (59), muscle (60,61), and heart (62,63). More recent studies have shown in mouse liver that phase contrast medium consisting of gas-filled microbubbles enhances gene delivery efficiency by increasing the cavitation of cell membrane (55,56). Since ultrasound is clinically used as a noninvasive diagnostic imaging tool and as a low-invasive method for shock wave treatment for urolithiasis, cholecystolithiasis, and choledocholithiasis, the technology would be extremely useful for nucleic acid delivery to the liver if the problem of low delivery efficiency and tissue damage associated with the procedure can be solved.

Hydrodynamic Delivery

In 1999, Liu *et al.* (65) and Zhang *et al.* (66) reported that a rapid injection of a large volume of DNA solution *via* the mouse tail vein delivered reporter gene into hepatocytes with high efficiency. This technique, more commonly called hydrodynamic delivery, is the simplest method for intrahepatic gene delivery. It involves an injection in 5–8 s of 8–10% of body weight in volume of isotonic DNA solution into the tail vein of a mouse. The mechanism of action underlying this procedure includes: (a) induction of cardiac congestion and elevation of pressure in the inferior vena cava; (b) retrograde flow of DNA solution into the liver; (c) enlargement of the fenestrae and generation of transient membrane defect on plasma membrane of hepatocytes; and (d) gene transfer into hepatocytes (67). Suda *et al.* have shown that the hydrodynamic impact on the liver is transient and reversible. It takes about 24–36 h for the liver endothelium to recover functionally and less than 1 min for the plasma membrane of hepatocytes to reseal (68). Using this method, 30–40% of the hepatocytes in the liver are transfected by a single tail vein injection of less than 50 μg of plasmid DNA into a mouse (65). Because of its simplicity, high efficiency, and reproducibility, hydrodynamic delivery has become a routine method for the delivery of DNA, siRNA, proteins, small compounds, and even viral vectors into the hepatocytes *in vivo* (69–73). Since its development in 1999, this procedure has been widely used for gene expression, gene knockdown, functional analysis of genetic elements, and establishing disease models in research animals (69,73).

While effective in rodents, hydrodynamics-based procedure has not been considered favorably for gene delivery in large animals or humans because injection of approximately 10% of body weight in volume (~7 L for a 70-kg man) is considered impractical and unsafe. However, the volume required can be reduced if a localized injection directly into the liver vasculature can be performed. For example, Eastman *et al.* have explored the possibility of hydrodynamic gene delivery through a catheter inserted into the hepatic vein under fluoroscopic guidance (74). They demonstrated in rabbits that a volume of 15 mL/kg can be safely injected to an isolated rabbit liver. Similarly, Kabayashi's group reported delivery of green fluorescent protein-containing plasmid DNA to the left lateral lobe of pig liver by catheterization and occlusion of the portal vein (75). With slight modification in procedure, Alino *et al.* (76), Fabre *et al.* (77), and Brunetti-Pierri (78) also demonstrated the feasibility for localized hydrodynamic delivery to the liver.

Realizing the intravascular pressure as the key parameter for hydrodynamic gene delivery to the liver, Suda *et al.* have recently developed a computer-controlled injection device aimed at the application of hydrodynamic delivery to humans (79). With a real-time feedback system based on vascular pressure to control the injection, the volume necessary for maximum effect of intrahepatic delivery was reduced from the original 10% of body weight to the current 5% for the entire liver of a mouse (79). By combining the computer-controlled device with image-guided catheterization technique, Kamimura *et al.* (submitted for publication) have recently established a procedure in swine for regional gene delivery to the liver. Assessments on cardiac function, tissue damage, and homeostasis revealed no abnormality on the animals tested (Kamimura *et al.*, submitted for publication). This image-guided, lobe-specific hydrodynamic procedure has great potential to become the method of gene delivery to the liver for human gene therapy.

FUTURE PERSPECTIVES

Nucleic acid-based therapeutics has been developed for the treatment of inherited and acquired diseases. The challenge, however, has been to develop a safe and effective method to bring therapeutic molecules into cells where treatment is needed. Biological, chemical, and physical principles are being utilized for the development of viral, synthetic, and physical methods, respectively. Significant progress has been made since the first gene therapy trial in 1990 (80) with respect to each type of vectors or a particular method. As far as the physical methods are concerned, the mechanisms of gene delivery by needle injection, ballistic bombardment (gene gun), electroporation, sonoporation, and hydrodynamics-based procedure have been fairly well-understood and all of these techniques are commonly used as a tool in research laboratories. With respect to their potential for liver-based clinic applications, however, hydrodynamic gene delivery appears to be most effective. Since its development in the late 1990s, this technique has become the routine method for liver transfection and been employed for the delivery of genes for gene therapy studies, siRNA for target validation, and viral genome for establishing HBV infection in mouse (69,73). The recent development of the computer-controlled injection device has made it possible for applying the hydrodynamic principle to gene delivery in large animals and possibly humans (79). Employing the image-guided catheterization technology to place the catheter to a specific site in a hepatic vein, one can now follow a standard procedure and perform site-specific hydrodynamic delivery not only to the liver but also to other organs (Kamimura *et al*., submitted for publication). To develop a clinically viable procedure for the liver, we will need participations of hepatologists who are trained to deal with liver diseases for establishing the optimal hydrodynamic parameters (injection speed, injection volume, pressure profiles) and for further improvements. With continuing efforts and the significant progress made in the past, it is foreseeable in the near future that the challenge for the development of a safe, effective, and clinically applicable method for nucleic acid delivery will be met. Consequently, the true value and benefit of nucleic acid-based therapy may be fully realized.

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