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TARGETING HEPATOCYTES FOR DRUG AND GENE DELIVERY: EMERGING NOVEL APPROACHES AND APPLICATIONS

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1. ABSTRACT

The asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes provides a unique means for the development of liver-specific carriers, such as liposomes, recombinant lipoproteins, and polymers for drug or gene delivery to the liver, especially to hepatocytes. The abundant receptors on the cells specifically recognize ligands with terminal galactose or N-acetylgalactosamine residues, and endocytose the ligands for an intracellular degradation process. The use of its natural ligand, i.e. asialofetuin, or synthetic ligands with galactosylated or lactosylated residues, such as galactosylated cholesterol, glycolipids, or galactosylated polymers has achieved significant targeting efficacy to the liver. There are several examples of successful targeted therapy for acute liver injury with asialofetuin-labeled and vitamin E-associated liposomes or with a caspase inhibitor loaded in sugar-carrying polymer particles, as well as for the delivery of a new antiviral agent, 9-(2-phosphonylmethoxyethyl)adenine. Liposome-mediated gene delivery to the liver is more difficult than to other organs, such as to lungs. It is still in its infancy due to difficulties in solving general issues, such as the circulatory stability of liposome-DNA complexes, and lysosomal or endosomal degradation of plasmid DNA. In spite of these existing concerns, several new approaches offer some reason for optimism, for example; intravenous injection of asialofetuin- or galactosylated cholesterol-labeled cationic liposomes has led to high transgene expression in the liver. In addition, specific antisense oligonucleotides against woodchuck hepatitis viruses incorporated into sialoorosomucoid-poly-L-lysine significantly inhibited viral replication in the liver. Finally, galactosylated polymers are promising for gene delivery, but require further studies to verify their potential applications.

2. INTRODUCTION

Hepatocytes are parenchymal cells in the liver which play major functions in many aspects of metabolism in the body, and are damaged in various pathological processes. Hepatocytes produce inflammatory mediators, such as free radical species and cytokines when damaged. These mediators initiate many pathologic cascades, such as fibrogenesis (1). Hepatocytes also produce a large number of serum proteins, and thus they are an attractive target for gene therapy to correct genetic defects, such as α1-antitrypsin deficiency, hemophilia, and lipoprotein receptor deficiency (2). Therefore, for attenuating liver injury, inhibiting hepatitis viral replication, or modifying hepatocyte-related metabolism, therapeutic agents should ideally be delivered to the liver, especially to hepatocytes.

To selectively target drugs and genes to hepatocytes using a variety of vehicles, such as liposomes, albumin, recombinant chylomicrons, lipoproteins, and microspheres, it is crucial that the vehicles are liver-specific, i.e. passive accumulation or active up-take of vehicles by the liver is highly preferential. For this purpose, liposomes are generated with a small diameter in order to pass through sinusoidal endothelial fenestrea, with negatively charged lipids, such as phosphatidylserine (PS) or phosphatidylinonsitol (PI), or shielded by carboxyl group-terminated amphiphilic polymers, such as branched poly(ethylene glycol) (PEG) (3). Liposomes containing dipalmitoylphosphatidylcholine (DPPC) are more rigid and are stable in serum. DPPC-liposomes have been shown to have better hepatocyte uptake and a shorter half-life in serum (4). Liposomes are also taken up by macrophages, or Kupffer cells in the liver, especially negatively charged liposomes because of CD36 or scavenger receptors on Kupffer and endothelial cells. The receptors recognize phosphatidylserine as a ligand for apoptotic cells (5). Thus, labeling liposomes with specific ligands or similar reagents to target specific receptors on hepatocytes is considered to be a promising approach to selectively deliver drugs or genes to hepatocytes (6). An abundant receptor specific to hepatocytes is the asialoglycoprotein receptor (ASGP-R), which recognizes and internalizes glycoproteins that have exposed terminal galactose or N-acetylgalactosamine residues (7). The present review covers the various approaches to enhance targeting efficiency of liposomes and other carrier systems to ASGP-R for drug or gene delivery to hepatocytes. Their delivery efficiency and therapeutic efficacy in the treatment of acute or chronic liver injury, hepatic fibrosis and viral hepatitis will be discussed. Some general-interest reviews on liposome-mediated drug and gene delivery have been published recently (8-9).

3. ASIALOGLYCOPROTEIN RECEPTOR

3.1. Biology of Asialoglycoprotein receptor

Asialoglycoprotein receptor (ASGP-R), also called hepatic lectin, is predominantly expressed on the sinusoidal surface of mammalian hepatocytes and is responsible for the clearance of glycoproteins with desialylated galactose or acetylgalactosamine residues from the circulation by receptor-mediated endocytosis. It also is responsible for the clearance of lipoproteins, and apoptotic cells. It is an integral transmembrane glycoprotein heteroligomer with an apparent molecular mass of 41 kD, which is composed of two structurally different subunits, H_1 and H_2, in human hepatocytes. H_1 is the major species of the receptor and is seven times more abundant than H_2. Both subunits are similar in molecular weight, and share 57% sequence homology (10). Subunit H_1 and H_2 have animo-terminal cytoplasmic tails, transmembrane domains that function as internal signal sequences, and carboxyl-terminal extracellular domains, which contain N-linked oligosaccharide binding sites. The galactose-binding extracellular domain belongs to the long-form subfamily with three conserved intramolecular disulphide bonds. It is able to bind terminal non-reducing galactose residues and N-acetylgalactosamine residues of desialylated tri- or tetra-antennary N-linked glycans. Subunit H_1 or H_2 deficient mice display phenotypic abnormalities in development and H_2 expression was abrogated in H_1-deficient mice and vise versa. The subunit deficient mice were unable to clear asialoorosomucoid (ASOR), a high affinity ligand for ASGP-R. However, there is no accumulation of desialylated glycoproteins or lipoproteins in the plasma (11). Copper and zinc ions at 0-225 μM reversibly blocked sustained endocytosis of isotope-labeled ASOR by 93% and 99% in isolated rat hepatocytes. Cells treated with copper and zinc lost their surface ASGP-R ligand binding activity up to 50% (12). In addition, chronic ethanol exposure leads to impairment of receptors in rat hepatocytes due to hyperphosphorylation of ASGP-R (13).

3.2. Carbohydrate recognition domain (CRD)

The specificity of the receptor for D-galactose or D-mannose is accomplished by specific hydrogen bonding of the 3 and 4-hydroxyl groups with carboxylate and amide side-chains. Therefore, mutation of the amino acid sequence in the carbohydrate recognition domain (CRD) results in a conversion of its specificity (14). The crystal structure of the CRD of H_1 subunit showed that three calcium ions form an integral part of the structure, which indicates its calcium-dependence for the carbohydrate-recognition. The structure also provides a direct confirmation for the conversion of the ligand-binding site of mannose-binding protein to an ASGP-R-like specificity (14). Moreover, a trimensional model for the human hepatic asialoglycoprotein receptor is proposed and can be used for study of the interaction of the ligand with specific amino acid residues, such as Trp and His residues in the recognition sites (10). A minimal 600 bp proximal region of the major subunit of the mouse ASGP-R exhibits hepatic-specific promoter activity in Hep G_2 cells (15). A functional mimic of the CRD has been developed by modification of the domain amino acid residues. The modified CRD displayed 40-fold preferential binding to N-acetylgalactosamine compared with galactose, making it a good functional mimic for ASGP-R (16).

3.3. Internalization of the ligands by ASGP-R

The internalization of the receptor-ligand complex occurs once the ligands bind to the extracellular domains of the receptors. The carbohydrate recognition domain binds to the specific carbohydrate residues in the extracellular space, and after endocytosis, the ligands are released into endosomes (lower pH). This process is pH-dependent (17). The acidification in endosomes leads to segregation of ligand from the receptor, with receptor molecules recycling back to the plasma membrane. Two subunits of the receptor are endocytosed at different average rates, and ligand binding increases the turnover rates of both subunits (18).

Besides binding to its specific ligands, rat ASGP-R also binds to the amino-terminal domain of thyroglobulin, thus binding is not oligosacharide-dependent. In man, the receptor is thought to be involved in the mediation of viral binding (preS1 attachment) to the hepatocytes, and the uptake of hepatitis B virus (HBV) (7, 19) and Marburg virus (20) during infection. Because the

receptors are expressed in most differentiated hepatocellular carcinoma (HCC) cells (21), this offers the possibility of selectively delivering chemotherapeutic agents to HCC in order to reduce their adverse effects on other tissues.

4. ASGP-R-MEDIATED DRUG DELIVERY TO HEPATOCYTES

The preferential existence of specific receptors on hepatocytes has made possible the selective targeting of therapeutic agents and foreign genes to hepatocytes. Over the past two decades, many attempts have been made to label liposomes or other carriers, such as polymers, human serum albumin (22), and recombinant high density lipoprotein (neoHDL) (23) with ASGP-R specific ligands, such as galactose, lactose, acetylgalactosamine and asialofetuin to develop hepatocyte-specific carriers for drug and gene delivery. In addition, synthetic galactose polymer ligands, such as poly-(N-?-vinylbenzyl-O-β-D-galactopyranosyl-[1-4]-D-gluconamide (PVLA), display a higher degree of affinity to ASGP-R than the natural ligand, asialofetuin. PVLA-coated beads were endocytosed by both cell lines expressing ASGP-R and by primary hepatocytes with a receptor-mediated process. Thus, the synthetic ligand may be a practical carrier-ligand for liver targeting (24). In the design of a receptor-specific ligand to label liposomes or other carriers, specific strategies have been employed to covalently attach a neoglycoprotein to the liposome surface to prepare neoglycoprotein-liposome conjugates with extended sugar epitopes by in situ enzymatic glycosylation, thus producing multisacharides on the surface (25). Potent trivalent cluster glycosides designed for the C-type ASGP-R provide an instructive example of how to turn the theoretical guidelines on ligand modification into nanomolar-affinity (25). Progress has been made to deliver antioxidants, such as vitamin E, or free radical scavengers, such as superoxide dismutase (SOD) (26-27), anti-viral agents, such as 9-(2-phosphonylmethoxyethyl)adenine (PMEA), as well as plasmid DNA with hepatocyte-specific liposomes, recombinant HDL, and other carriers.

4.1. Asialofetuin-labeled liposomes

Asialofetuin (AF) is a natural ligand for ASGP-R, and processes triantennary complex carbohydrate chains. Its receptor dissociation constant is 200 fold lower than the glycoproteins with biantennary N-linked oligosaccharide chains (28). This implies that asialofetuin binds the ASGP-R with high affinity. We chose asialofetuin to label conventional liposomes which were used in our previous study (27), and found that labeling the conventional liposomes with asialofetuin significantly enhanced liver up-take of the AF-labeled liposomes from 16.5 ± 1.8% to 73% ± 3.9 % during the first 4 hours after the injection. Much more fluorescent signals were detected in liver parenchymal cells from mice injected intravenously with 1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled AF-liposomes than the conventional liposomes. These lines of evidence suggest that asialofetuin is a useful protein in enhancing liver up-take of the liposomes, preferentially by hepatocytes (29). Moreover, vitamin E-associated AF-liposomes displayed better protection against acute liver injury induced by carbon tetrachloride (CCl_4) challenge in mice than those without AF labeling, and in fact, the alanine aminotransferase (ALT) levels in the group treated with CCl_4 plus VE-AF-liposomes were close to normal controls (29). Our results were further supported by an observation that galactose-tagged conventional liposomes generated from n-glutary-phosphatidylethanolamine displayed a higher ratio of in vivo liposomal incorporation into parenchymal (P) cells versus non-parenchymal (NP) cells, and that distearoyl phosphatidylethanolamine conjugated with galactosylates sterically stabilized with PEG (PEG-DSPE) further enhanced the ratio of P:NP to 97:7. Mannose-labeling shifted the ratio to more non-parenchymal cell incorporation (the majority to Kupffer cells) (30). However, AF is a glycoprotein that may elicit antigenicity when administrated to animals or to man; therefore alternative approaches are needed to target liposomes to hepatocytes via ASGP-R.

4.2. Galactosylated cholesterol

One approach that has been tried by two groups is to conjugate galactose with cholesterol (Chol). Cholesterol is a neutral lipid and is often used in the generation of various types of liposomes, such as cationic (DOTAP-Chol) for gene delivery, anionic (phosphatidyl-serine-Chol) and neutral liposomes (phosphatidylcholine-Chol) as a co-lipid. Kawakanmi et al. synthesized a galactosylated cholesterol derivative, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiogalactosyl-ethyl)amino)butyl) formamide (Gal-C_4-Chol) (31), that has been found useful for both drug and gene delivery to the liver (32-33). Liposomes generated from distearoyl-phosphatidylcholine (DSPC) and Gal-C_4-Chol were employed to deliver prostaglandin E_1 (PGE_1) and probucal in mice. It was found that 85% of DSPC-Gal-C_4-Chol liposomes accumulated in the liver 10 min after intravenous injection and that the ratio of the liposome distribution in parenchymal cells and non-parenchymal cells in the liver was 15.1:1 in comparison with 1.1:1 when DSPC-Chol liposomes were injected as controls. PGE_1 and probucol were efficiently delivered with 50% recovery in the liver (32-33).

Another group developed a series of glycolipids consisting of cholesterol and a cluster galactoside moiety with an ester spacer to link the lipid and galactosides (34). The glycolipids were incorporated into liposomes generated from egg yolk phosphatidylcholine and cholesteryl oleate in a 5% concentration (w/w). More than 80% of the injected liposomes accumulated in the liver 30 min after intravenous injection of these galactoside-loaded liposomes. Pre-injection of asialofetuin significantly inhibited the liver up-take of galactoside-loaded liposomes. The advantage of these amphiphilic galactoside-loaded liposomes is that water-soluble substances can be encapsulated in the liposomes for efficient delivery to parenchymal cells in the liver. Previously a low encapsulation rate of water-soluble substances in neutral liposomes has limited their wide-spread application in drug delivery.

4.3. Soybean-derived sterylglucoside

Soybean-derived sterylglucoside (SG) is a residue extracted from soybeans. Liposomes generated from dipalmitoyphosphatidylcholine (DPPC), cholesterol and SG at a molar ratio of 6:3:1 have been shown to lead to a high liver accumulation in mice (35). This formulation of liposomes was used to entrap a chemotherapeutic agent, doxorubicin, and the liposome-mediated doxorubicin incorporation in Hep G_2 cells was investigated. It was found that the incorporation of doxorubicin into the cells after incubation with DPPC-Chol-SG-doxorubicin liposomes was much higher than with liposomes devoid of SG. This suggests that the DPPC-Chol-SG liposome-mediated doxorubicin delivery into cells is probably through ASGP-R-mediated endocytosis. Thus, SG may work as a potential ligand to label liposomes for hepatocyte targeting, and SG-liposomes are potentially useful drug carriers to parenchymal cells in the liver (36). However, additional animal experiments are needed to verify their drug targeting efficiency.

4.4. Recombinant high density lipoprotein (neoHDL)

The main function of high density lipoprotein (HDL) in the circulation is believed to be the removal of excess cholesterol from extrahepatic or peripheral tissue followed by transport to the liver. This process is known as “reverse cholesterol transport”. This “homing” transport is a receptor-recognizing process, and natural HDL recognizes the apoE-specific remnant receptor in hepatocytes. Recombinant spherical HDL (neoHDL), constructed of lipid and lactosylated apolipoprotein has been shown to be able to specifically target to the ASGP-R on parenchymal cells (37). Therefore, lactosylated neoHDL (Lac-neoHDL) may represent a potential carrier system for the delivery of lipophilic (pro)drugs to the parenchymal liver cells, especially since lipophilic prodrugs can be incorporated into the lipid moiety in the particles without interfering with the receptor-mediated recognition of the lactosylated apolipoproteins. 9-(2-phosphonylmethoxy-ethyl)adenine (PMEA) is a new effective agent against hepatitis B virus (HBV) in vitro. However, there is very little up-take by the liver in vivo, and it is largely excreted by the kidneys. The therapeutic dose window between inhibition of viral replication and renal toxicity is small. Therefore, a targeted delivery system may change its pharmacokinetics, enhance its liver distribution and reduce its renal toxicity. PMEA was first coupled to di- and trivalent cluster glycosides, i.e. $K(GN)_2$ and $K_2(GN)_3$, respectively with nanomolar affinity for C-type ASGP-R (25) to yield a hepatotrophic prodrug. Liver uptake of the prodrug was enhanced by 10-fold in comparison with the parent drug and 90% of the prodrug was localized in liver parenchymal cells (38). In addition, the antiviral activity of the prodrug was enhanced by 5- and 52-fold for $K(GN)_2$-PMEA and $K_2(GN)_3$-PMEA. This approach represents one successful method of targeting delivery through ASGP-R. In another study, PMEA was incorporated into the lipid moiety of Lac-neoHDL by attaching, via an acid-labile bond, lithocholic acid-3α-oleate (LO) to the drug. The incorporation of the prodrug to the Lac-neoHDL markedly increased the up-take of lipophilic prodrug PMEA-LO by the liver. 68±7.7% of the injected dose was localized in the liver 30 min after the intravenous injection and 88.5% of PMEA-LO was detected in the parenchymal cells. Furthermore, asialofetuin pretreatment markedly inhibited the parenchymal incorporation and total liver up-take. The prodrug was processed in the lysosomes after being taken up by the parenchymal cells and PMEA was released into the cytosol, where it was converted into its active diphosphoralylated metabolite (39). A separate in vitro experiment documented that the lactosylated neoHDL-associated PMEA-LO much more efficiently inhibited HBV replication in a HBV-transfected Hep AD38 cell line (35 times lower in viral titer) than free PMEA (40). Thus, lactosylated neoHDL is a useful carrier for delivering lipophilic drugs to the liver, especially hepatocytes (23). Both cluster glycosides and lactosylated neoHDL are promising carriers for changing PMEA pharmacokinetics, reducing the renal toxicity, and for enhancing liver distribution.

4.5. Other drug delivery systems

Nanometer particles, generated from poly (γ-benzyl L-glutamine) (PBLG) or poly(lactic acid) (PLA), were conjugated with poly(vinyl benzyl-lactonamide) (PVLA) as the carbohydrate-carrying polystyrene (PS). The particles were loaded with colchicine, cytochalasin B and taxol for testing their feasibility in delivering the drugs to primary hepatocytes in vitro. It has been shown by confocal microscopy that the drug-loaded particles coated with sugar-carrying polymers were internalized by the hepatocytes after one hour of incubation, and that the internalization process occurs via a receptor-mediated mechanism (41). A PLA nanocarrier was used to deliver a caspase inhibitor, Z-Asp, in a mouse model of acute hepatitis induced by concanavalin A intravenous injection, a prominent feature of hepatocyte apoptosis. It was documented that nanocarrier-encapsulated Z-Asp extended the intracellular retention time of the drug in hepatocytes, and that by modifying the components of nanocarriers, it was possible to control the release rate of the entrapped content from the nanocarriers. The nanocarriers with controlled degradation rescued mice with lethal hepatic injury (42). This temporally and spatially controlled drug delivery system could be used in a variety of liver diseases.

5. HEPATOCYTE-SPECIFIC CARRIERS FOR GENE DELIVERY

5.1. General aspects of liposome-mediated gene delivery

Non-viral vectors for gene delivery include liposomes (cationic or anionic), polymers and DNA condensing proteins or binding molecules. Cationic liposomes are the most widely used non-viral vectors for in vitro gene transfection and have been investigated for in vivo gene delivery to treat lung cancers and metastatic liver tumors (43 44), as well as a genetic deficiency in bilirubin metabolism (45). Compared to drug delivery, liposome-mediated in vivo gene delivery is still in its infancy and many issues that affect its delivery remain to be solved as illustrated in Figure 1. The main issues include: 1) the formation of aggregates between cationic lipids and serum proteins bearing negative charges (albumin, lipoprotein, etc.); 2) the administration routes of liposome-DNA

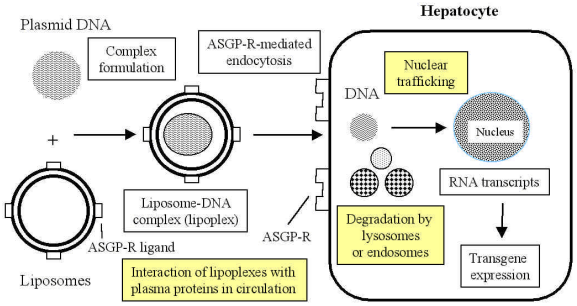

Figure1. Schematic illustration of mechanisms and barriers in liposome-mediated gene delivery to hepatocytes. Potential barriers are highlighted with dark background. Lipoplexes formed from specific ligand-labeled cationic liposomes and plasmid DNA are administered through either peripheral vein or portal vein. In both administration routes, the lipoplexes may interact with plasma proteins, which results in large aggregates. The aggregates may be large enough to be trapped in the lung circulation and only small part of injected lipoplexes will reach the liver when the lipoplexes are administrated intravenously. Lipoplexes are endocytosed by hepatocytes when they have been recognized by the cells. Endocytosed lipoplex components including plasmid DNA may undergo degradation by catalytic enzymes in lysosomes or endosomes, and only a small portion of DNA is able to enter the nucleus through the nuclear pore complex. Episomal DNA in the nucleus will be transcribed, and transgene expression can be detected in cell lysates. ASGP-R = asialoglycoprotein receptor.

complexes (lipoplex); 3) intracellular trafficking from cytoplasm to nucleus; 4) the proliferation state of cells to be transfected; and 5) transient transgene expression. Substantial efforts have been made to address these problems (9). These efforts include: a) the incorporation of amphiphilic PEG into cationic lipids to shield the charges of liposome-DNA complexes (lipoplex) and to provide a means of steric protection (46); b) the use of poly-L-lysine, nuclear proteins or viral proteins to condense the plasmid DNA (18; 47-48); and c) the use of intracellular receptors to promote translocation of plasmid DNA from the cytoplasm to the nucleus (49). In another study, fusigenic viral envelope proteins were incorporated into cationic liposomes to form virosomes (HVJ liposomes), which may have higher integration of transgenes than liposomes (50).

Progress has been made to reduce unwanted aggregates of cationic lipids with serum proteins. We have recently described methods for the polymerization of a novel cationic acrylamide lipid and reconstitution of the resultant poly(cationic lipid) (PCL) to yield stable cationic vesicles (51). We have studied extensively the toxicity, stability and gene transfection of the polymerized cationic liposomes generated from PCL and cholesterol (PCL-Chol) (51). Our findings demonstrated that PCL and PCL-Chol are serum-resistant since size distribution of the liposomes did not change significantly when exposed to high serum concentration in culture medium for a prolonged period. The serum-resistant PCL and PCL-Chol also have little or no cytotoxicity to hepatocytes and Hep G_2 cells, and display a transfection efficiency in hepatoma cell lines similar to commercially available agents, such as Lipofectamine® (51). This formulation of polymerized liposomes was also shown to have much less protein binding in vivo in comparison with DOTAP-Chol or DOTAP-DOPE 30 minutes after they were injected intravenously (unpublished data, Wu et al). This PCL-Chol formulation has been shown to be very effective in the delivery of reporter genes (green fluorescent protein and luciferase) to the liver when the liposome-DNA complexes (lipoplexes) are administrated via the portal vein in mice. The transgene expression in the liver has been markedly enhanced by prior partial hepatectomy or subcutaneous injection of thyroid hormone (triiodothyronine, T_3) (52). We also explored a route of administration of lipoplexes through an indwelling catheter in the portal vein for multiple injections. The combined efforts, such as the non-invasive T_3 pretreatment and indwelling catheter in the portal vein, achieved an extended transgene expression in the liver at a high level (unpublished data, Wu et al.). We speculate that our serum-resistant formulation of polymerized cationic liposomes may also have an improved targeting efficiency once they are formulated with a hepatocyte-specific ligand, such as Gal-C_4-Chol.

5.2. Liver-specific cationic liposomes labeled with either asialofetuin or Gal-C_4-Chol

Asialofetuin (AF) was employed to label cationic liposomes containing N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride, and AF-labeled liposomes were complexed with plasmid DNA using a bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Hep G_2 cells and young rat hepatocytes with stimulation of epidermal growth factor and insulin were transfected successfully with the lipoplexes. The transfection efficiency in the hepatoma cell line was two times higher than in non-labeled liposomes (53-54). Asialoorosomucoid-labeled cationic liposomes with a different formulation were also shown to be more effective in transfection of Hep G_2 cells than liposomes without labeling (55). Although portal vein injection of AF-liposome-DNA complexes led to significant transgene expression in liver parenchymal cells, there has been no systemic administration experiment available to confirm the targeting gene delivery efficiency of this formulation of AF-liposomes (56). However, DOTAP-Chol liposomes coated with succinylated AF did lead to a 7-fold increase in CAT expression in mouse liver 24 hours after tail vein injection of the lipoplexes, in comparison with the liposomes without AF labeling (57).

Because of the successful, targeted delivery of lipophilic substances by neutral liposomes generated from distearoylphosphatidylcholine (DSPC) and Gal-C_4-Chol (31), Gal-C_4-Chol has been used to generate cationic liposomes for gene delivery. Cationic liposomes generated from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride (DOTMA)-Chol-Gal-C_4-Chol displayed higher transgene expression in the liver in comparison with DOTMA-Chol when both types of lipoplexes were administrated through the portal vein. There was preferential transgene expression in liver

parenchymal cells following the injection of lipoplexes comprised of DOTMA-Chol-Gal-C_4-Chol. The transgene expression was inhibited by prior administration of galactosylated bovine serum albumin (BSA) (32). Therefore, it appears that the incorporation of Gal-C_4-Chol in cationic liposome formulations enhances the transgene expression in the parenchymal cells only when the lipoplexes are administrated through the portal vein. For efficient targeting of hepatocytes through systemic administration, a better cationic liposome formulation, one which would have less interaction with plasma proteins and less loss during first pass through the lung circulation after peripheral administration, is needed.

5.3. Asialoorosomucoid-conjugated poly-L-lysine

Poly-L-lysine in the presence of a high concentration of NaCl (0.7-0.75 M) interacts with DNA, and fully condenses DNA molecules when there is an excess of DNA in the system (58). Poly-L-lysine-condensed DNA is more resistant to nucleases in plasma. Asialoorosomucoid (ASOR) is another natural ligand for ASGP-R, which has been used to conjugate to poly-L-lysine for targeting antisense oligodeoxynucleotide, to inhibit HBV replication in a cell line and woodchuck hepatitis virus (WHV) in vivo (18, 59). The antisense DNA oligonucleotides against the polyadenylation region of the WHV gene was complexed with ASOR-poly-L-lysine and injected intravenously into chronically infected animals (0.1 μg/kg/day) for five consecutive days. A 5- to 10-fold decrease in circulating WHV-DNA was observed in treated animals 25 days after the treatment (60). Animals treated with complexed random DNA or with antisense DNA alone showed no decrease in DNA levels, indicating that only appropriate antisense sequences in a complex produced the observed inhibitory effects.

5.4. Galactosylated Polymers

Various types of polymers have been tested for gene delivery. Galactosylated polyethylenimine (PEI) has been employed for in vitro gene transfer to hepatocytes (61) and for delivering a functional gene to the liver (62). Poly(L-ornithine) (pOrn) was first modified with galactose, then with a fusigenic peptide (mHA_2) to obtain Gal-pOrn-mHA_2 for complexing with plasmid DNA. The Gal-pOrn-mHA_2-DNA complexes were injected intravenously to target hepatocytes in mice. The polymer-DNA complexes (polyplexes) are 100-150 nm in size, and seem to be more effective in delivering a reporter gene to hepatocytes as indicated by much higher (one to two log level) transgene expression in the liver than DNA-pOrn, DNA-Gal-pOrn and DOTMA-Chol-DNA (63). In another report, a synthetic peptide, Cys-Trp-Gys_{18} (CWK_{18}), which forms small DNA condensates, is capable of mediating efficient non-specific gene transfer to cells in culture. Based on this result, a natural triantennary N-glycan ligand has been covalently attached to the side chain of cysteine in CWK_{18}, resulting in a triantennary glycopeptide (Tri-CWK_{18}) that binds to the ASGP-R with a nM dissociation constant. Tri-CWK_{18} was further coupled to PEG to form optimal DNA condensates with specific targeting properties to ASGP-R on mammalian hepatocytes. Then Tri-CWK_{18}-PEG was employed to deliver the human α1-antitrypsin gene to mouse liver through peripheral intravenous injection. 80% of the plasmid DNA was found in the parenchymal cells 2 hours after the injection of 50 μg plasmid DNA with Tri-CWK_{18}-PEG, and there were detectable levels of human α1-antitrypsin in the mouse serum which peaked at 7 days after the injection (64).

Poly(2-(dimethylamino)ethyl methacrylate (DMAEMA)-*co*-*N*-vinyl-2-pyrrolidone (NVP) was conjugated with PEG and further coupled with a galactose moiety at the PEG terminal end to form poly(DMAEMA-NVP)-*b*-PEG-galactose for targeting gene delivery to hepatocytes (65). When this polymer formulation is complexed with plasmid DNA, the resultant polyplex exhibited a negative charge. Then the polyplex was coated with the cationic, pH sensitive, endosomolytic peptide, KALA to generate positively charged poly(DMAEMA-NVP)-*b*-PEG-galactose/DNA/KALA complex particles. These particles displayed similar transfection efficiency in Hep G_2 cells as Lipofectamine plus. Further study is needed to verify in vivo targeting efficacy of the sophisticated polymer complexes (65).

In summary, although ASGP-R offers a specific targeting approach for the development of hepatocyte-specific liposomes or other carriers, the receptor-mediated endocytosed liposomes and their entrapped genetic material may undergo lysosomal or endosomal degradation. Thus, special attention should be focused on how to enhance the intracellular trafficking of endocytosed genetic materials to the nucleus. Using DNA condensates or employing intracellular receptors, such as steroid receptors, may offer new solutions to abrogate the intracellular degradation.

6. CONCLUSIONS AND PERSPECTIVES

The present review highlights new understandings of asialoglycoprotein receptor-mediated endocytosis and new developments in the use of this abundant receptor for targeted delivery of drugs and genes to hepatocytes. Novel hepatocyte-specific carriers, such as liposomes, recombinant high density lipoproteins, and polymers have been developed for selective delivery of therapeutic drugs, such as vitamin E, or anti-viral or chemotherapeutic agents to the liver. For liver-specific gene delivery, non-viral vectors, such as galactosylated liposomes and polymers are promising and feasible carriers. Further research should focus on developing new serum-resistant carriers, such as poly(cationic lipid) to enhance their stability in the circulation, DNA condensates such as poly-L-lysine, Cys-Trp-Gys_{18} (CWK_{18}) or viral and nuclear proteins, such as adenovirus hexon protein, protamine sulfate, and histone H_1 (9, 47-48) to prevent DNA degradation in the plasma or cytosol. Preventing lysosomal or endosomal degradation of endocytosed genetic materials, and enhancing their trafficking from the cytosol to the nucleus are crucial factors in improving ASGP-R-mediated gene delivery and transgene expression. For this purpose, intracellular receptors, which are translocated into nuclei after binding to their ligands are of special interest. Thus, hepatocyte-specific carriers for selective delivery of therapeutic genetic material to the

liver may well be available in the near future. The hope is that targeted therapeutics will benefit patients with liver disorders in the near future.

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Abbreviations: AF = asialofetuin; ASGP-R = asialoglycoprotein receptor; CAT = chloramphenicol acetyltransferase; Chol = cholesterol; CRD = carbohydrate recognition domain; DOPE = L-α dioleoyl phosphatidylethanolamine; DOTAP = 1,2-bis(dioleoyloxy)-3-(trimethylamonio)propane; DPPC = dipalmitoylphosphatidylcholine; DSPC = distearoyl-phosphatidylcholine; GFP = green fluorescent protein; HBV = hepatitis B virus; HCC = hepatocellular carcinoma; neoHDL = recombinant HDL; PCL = poly(cationic lipid); PEG = polyethylene glycol; PEI = polyethylenimine; PEG = poly(ethylene glycol); SG = soybean-derived sterylglucoside; T_3 = triiodothyronine

Key words: Asialofetuin, Asialoglycoprotein receptor; Carbohydrate recognition domain; Drug delivery, Hepatocyte, Gene therapy, Liposome, Liver, Polymers, Targeting therapy, Review

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