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TARGETING KUPFFER CELLS WITH ANTISENSE OLIGONUCLEOTIDES

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1. ABSTRACT

During proinflammatory reactions such as endotoxemia, ischemia-reperfusion and immune reactions, excessive amounts of cytokines and prostanoids are released resulting in liver injury. In the liver, Kupffer cells are the primary source of cytokines and prostanoids. Obliteration of Kupffer cells prevents experimentally-induced liver damage, suggesting a major role for Kupffer in the pathogenesis of liver diseases. Pretreatment of experimental animals with antibodies directed against cytokines such as tumor necrosis alpha (TNF-alpha) prevented experimentally-induced liver damage. In recent years, antisense oligonucleotides (ASOs) directed against specific mRNAs have been tested as an alternative therapy to control the excessive production of inflammatory peptides. Although ASOs have great potential against gene expression, their design, *in vivo* delivery and stability, have posed significant challenges. Computer-aided configurational analysis and identification of viable motifs (GGGA) on the pre-mRNA transcripts have, in part, alleviated the problems in designing effective ASOs. However, the major challenge involves the *in vivo* delivery of an ASO to the target tissue. Additionally, it is critical that once taken up by the cells, the ASO is able to survive the lysosomal barrier and enter the cytoplasm. Anionic liposomes and lactosylated low-density lipoproteins (LDL) have been successively used as adjuvants for delivery of ASOs to Kupffer cells. In particular, pH-sensitive liposomes have shown great promise as delivering vehicles to target Kupffer cells. In summary, although ASOs are emerging as a new class of drugs against Kupffer cell-derived pro-inflammatory molecules, *in vivo* delivery still remains a challenge. pH-sensitive liposomes and LDL-

based delivery systems show significant promise for specifically targeting Kupffer cells.

2. INTRODUCTION

Liver comprises both parenchymal and non-parenchymal cells. Hepatocytes constitute the parenchymal cells whereas Kupffer cells, endothelial cells and Ito cells (also called fat-storing cells or stellate cells) correspond to the non-parenchymal cells. Hepatocytes account for about 65% of the liver cells and 90% of liver mass. Kupffer cells, the resident macrophages in the liver, which account for about 15% of liver cells, constitute less than 3% of the liver cell mass. Nevertheless, these macrophages play a critical role in the pathogenesis of liver diseases. Kupffer cells belong to the mononuclear phagocyte family of cells and are recruited from the stem cells of bone marrow (1). After a series of intermediary stages, they differentiate into tissue macrophages and remain embedded in the liver sinusoids. These liver macrophages are in close contact with sinusoidal endothelium and may have extensions to reach the parenchymal cells as well. Thus, the Kupffer cells are positioned to influence the biochemical and physiological events in the neighboring cell types. Kupffer cells constitute the largest single pool of macrophages in the body (2). The strategic position of these macrophages within the liver sinusoids makes them the first macrophage cell type to come into contact with foreign bodies such as viruses and bacteria that enter circulation via the portal vein. Being a part of the immune system, Kupffer cells are activated, and release cytokines such as tumor necrosis factor (TNF-alpha), interleukin-1 (IL-1), IL-6, and prostanoids as part of the defense mechanism to neutralize the challenge (3). However, as described below, an over expression of these pro-inflammatory chemicals, result in liver damage.

3. KUPFFER CELLS AND LIVER DAMAGE

One of the key elements associated with liver injury is the production of reactive oxygen species (ROS). It has been reported that the phagocytic function of macrophages depends, to a large extent, upon the production of ROS such as, superoxide anions, hydrogen peroxide and other oxygen radicals mediated by NADPH-oxidase (4). Although Kupffer cells play a mainly protective and pro-regenerative role in the liver (5,6), they also play a role in liver injury, most likely due to an excessive release of proinflammatory cytokines and prostanoids, such as during endotoxemia, hypoxemia and immune reactions (7-9). The role of Kupffer cells in the generation ethanol-induced oxidative stress has been recently described by Bautista and Spitzer (10). The ability of Kupffer cells to secrete cytokines and prostanoids under various circumstances has been described in detail by Decker (3). A direct role of Kupffer cells in liver damage was found in studies in which deprivation of Kupffer cells prevented liver damage induced by endotoxin, alcohol and acetaminophen in experimental animals (11-13). Recent studies by Thurman and coworkers (14,15) have demonstrated that NADPH-oxidase plays a vital role in alcohol-induced liver injury and that *in vivo* delivery of superoxide dismutase (SOD) ameliorates hepatic injury. Similarly, in the acetaminophen-induce liver injury model, *in vivo* delivery of SOD prior to acetaminophen treatment prevented liver injury (16). In their study, SOD was encapsulated in large-sized liposomes and Kupffer cells were the primary targets. The role of NF-kappa B, as a key intermediate in the sequence of events that link liver injury and oxidative stress has been discussed by Wu and Zern in a carbon tetrachloride (CCl_4)-induced model of liver injury (17). They have also demonstrated that *in vivo* delivery of antioxidants such as Vitamin E reduced CCl_4-induced liver injury.

Among the proinflammatory agents, tumor necrosis factor alpha (TNF-alpha), a pleiotropic cytokine, has taken the role of a key player in the cascade of events that result in tissue injury (18). However, it is not clear whether hepatic injury is caused directly by the ROS or is mediated or amplified through TNF-alpha-mediated mechanisms. A likely mechanism is the generation of TNF-alpha mediated by oxidative stress via NF-kappaB, which would explain both, the effects of radical scavengers, SOD and factors that reduce TNF-alpha. Antibodies against TNF-alpha have been shown to prevent experimentally-induced liver damage (19,20). Nevertheless, toxicity issues limit the therapeutic use of these antibodies in humans (18). Against that background, the possibility of using antisense oligonucleotides (ASOs) as an alternative approach to suppress the production of proinflammatory peptides has been gaining attention in recent years (21,22). In fact, some twenty ASOs are undergoing phase III clinical trials to treat various diseases (www.recap.com).

4. ANTISENSE OLIGONUCLEOTIDES

4.1. Background

In 1978, Zamecnik and Stephenson were the first to observe that oligonucleotides complementary to specific regions of viral mRNAs inhibited *in vitro* replication (23). Antisense oligonucleotides are short DNA molecules, generally 15-30 nucleotides long. The specificity of ASOs is due to a highly selective hybridization of the oligomers to their complementary target sequence on the mRNA, resulting in the inhibition of protein expression by at least two widely accepted mechanisms. First, the ASOs form a DNA-RNA hybrid with the pre-mRNA transcript (in the nucleus) promoting the hydrolysis of RNA by RNase H (24). Second, depending upon the region of the mRNA targeted, ‘exons’ or ‘introns’, the ASOs can also bind to the mRNA in the cytosol and prevent translation. In the latter mechanism, a reduction in protein synthesis may occur without a change in mRNA levels. The mechanisms of antisense action have been recently reviewed (25). The ability of the ASOs to suppress specific gene expression has created a therapeutic potential for the ASOs in the treatment of cancer, viral infections and other diseases (26). However, the actual challenge lies in our ability to deliver the ASO to the target tissue, and then into the appropriate compartment in the cell, so that maximum efficacy can be achieved at the lowest concentration.

4.2. Design of antisense oligonucleotides

There are several considerations in designing an antisense molecule; stability, solubility, specificity and efficacy. Since the unmodified phosphodiester

oligonucleotides are vulnerable to nuclease attack and have very short biological half-lives, a more stable, phosphorothioate-modified ASO (P-ASO), in which one of the oxygen atoms in the phosphodiester linkage is substituted by a sulfur atom, was developed as the first generation of modified ASOs (27). The *in vivo* half-life of P-ASO is about 48 h (28-30) as opposed to a much shorter time for the unmodified ASO (31). Although second and third generation of ASOs have been developed by substitutions on the phosphate moiety and the use of substituted ribonucleotides, each ASO type has its advantages and disadvantages with respect to binding properties, solubility and toxicity (26). Nevertheless, the P-ASOs, because of their high solubility in water have been widely used. One of the major problems of ASO design is the selection of appropriate target sites on a given mRNA transcript. Generally, only 5-6% of the antisense molecules generated against the transcript are effective (32). Factors such as binding energy and $\Delta G_{formation}$ for ASO-mRNA hybrids were thought to play a role in the selection of an ideal ASO molecule (33,34). However, these approaches did not provide a clear-cut strategy for designing an effective ASO. Recently, after an exhaustive survey of literature, Tu et al (21) from our laboratory, made an observation that about 50% of the ASOs that were effective, unwittingly contained a TCCC sequence that was complementary to GGGA motif on the target mRNA transcript. To check the validity of this observation, Tu et al (21) constructed a number of 18-21 mer P-ASOs incorporating the 5'TCCC3' motif to anneal to the 5'GGGA3' sequence using TNF-alpha mRNA as the target molecule, and determined the inhibitory property of the ASOs against the LPS-induced production of TNF-alpha by primary cultures of rat Kupffer cells. They observed that 60% of the TCCC-containing ASOs were effective. The lack of inhibition among 40% of the ASOs may suggest that secondary structures, in addition to the GGGA motif may play a role in the efficacy of ASOs. These studies provided some resolution to the problem of designing effective ASOs without having to target the entire mRNA molecule, sequence by sequence. However, on a cautionary note, it is important to avoid CpG sequences in the ASOs. The presence of unmethylated CpG motifs elicits an immune reaction, and its mechanisms have been recently reviewed (35).

4.3. Delivery of ASOs

The most challenging aspect of using ASO therapeutically lies in our ability to deliver the molecule to its final destination, in this case, the cytosol or the nucleus. For *ex-vivo* cell culture systems, there are two major barriers; the plasma membrane and the endosomal/lysosomal barrier. In the latter case, once the ASO is released from the endosomal/lysosomal sac, the cytosolic ASO can diffuse into the nucleus. Indeed, ASOs that are directly injected into the cytosol have been shown to diffuse into the nucleus (36). However, *in vivo* delivery is much more challenging; factors such as immunomodulation, complement activation, stability in serum, and the phagocytic challenges of the reticulo-endothelial system (RES) have to be overcome before the ASO is presented to the target cells, details of which will be discussed later in this section. Several ASO delivery systems have been developed, each having its merits and demerits. For an extensive review of the various antisense oligonucleotide delivery systems please see the review by Garcia-Chaumont et al (37). However, in this section we will provide an overview of some of the *ex-vivo* and *in-vivo* delivery systems that have either been used or have the potential to be used for the delivery of ASO to macrophage/macrophage-type cell systems. Although, as indicated earlier, ASOs with many different backbone modifications exist, most of the studies reported here have used the phosphorothioate-modified ASO, the most commonly used ASO type.

4.3.1. *Ex vivo* delivery of ASOs

The primary objective of delivery of ASO in *ex-vivo* studies is to determine the efficacy of a given ASO construct against the targeted gene product. Therefore, the possible whole animal toxicity of the delivery systems is not relevant. However, once the efficacy of the ASO is established, the next step is to search for a suitable *in vivo* delivery system, which, as it will be discussed later, is more complex.

4.3.1.1. 'Naked' ASOs

Although the simplest way to treat the cells is to add the ASO directly into the medium, this method is the least efficient way to deliver an ASO. It has been reported that it takes almost 10 times higher concentration of 'naked' ASOs to induce the same effect as compared to vector-mediated delivery systems (37). By contrast, as will be discussed later, 'naked' ASOs are efficiently taken up by cells *in vivo*.

4.3.1.2. Liposomal delivery systems

4.3.1.2.1. Anionic liposomes

Unlike 'naked' ASOs where the mechanism of cell entry is unclear, liposomal delivery takes place essentially by the endocytic pathway. There are two categories of liposomes, the 'anionic' and the 'cationic' types. Anionic liposomes are lipid vesicles, essentially consisting of phospholipids and cholesterol (in which, the ASO is encapsulated). Anionic lipids are considered safe, and are approved by the Food and Drug Administration. Anionic liposomes containing phosphatidyl serine (38,39) or cardiolipin (40) have been used for ASO delivery in cultured cells. However, a problem with anionic liposomes is that the encapsulation efficiency is low, requiring the use of relatively high concentrations of the lipid. Also, if they are pH-insensitive, following internalization, most of the ASOs are bound to remain lodged in the endosomal/lysosomal compartment, to be eventually degraded by the hydrolytic enzymes. The issue of destabilizing the endosomal compartment was addressed using pH-sensitive liposomes. These are anionic liposomes that contain phosphatidylethanolamine (PE), an amphipathic lipid that changes its charge at slightly acidic pHs. These lipids form a stable bilayer at physiological pH only in the presence of titrable acids such as cholesterylhemisuccinate (CHEMS) or oleic acid (41,42). Once endocytosed, these liposomes get protonated in the acidic endosomes/lysosomal compartment and destabilize

the endosomal membrane, allowing the ASOs to be released into the cytosol. As mentioned above, once in the cytosol, they are rapidly sequestered into the nucleus. The efficacy of these liposomes has been demonstrated in cell culture systems (43-45). Encapsulation efficiency can be improved by using the reverse phase method (46) originally described by Szoka and Papahadjopolous (47) for pH-insensitive liposomes. However, with the advent of cationic liposomes, the use of anionic liposomes for *ex vivo* studies has become limited. It should however be noted that cationic liposomes are destabilized by serum (*vide infra*).

4.3.1.2.2. Cationic liposomes

Cationic liposomes essentially consist of a cationic molecule such as DOTMA (N-[1-(2,3-diolexy)propyl]-n,n,n-trymethylammonium chloride) and a phospholipid DOPE (Dioleyl-PE) (48), commercially available as Lipofectin™. ASOs, being negatively charged at physiological pH, electrostatically interact with cationic liposomes. An association of ASOs with cationic liposomes can be achieved by a simple mixing of the two components, and represents an advantage over the more complex and time-consuming procedure of preparing anionic liposomes. The high efficacies of using cationic liposomes *ex vivo* in the delivery of ASOs in Kupffer cells (21) and other cell types have been demonstrated (49,50; also, see ref. 37 for the various types of cationic lipids used in ASO delivery). To date, cationic liposomes constitute the most efficient method of delivering ASOs for *ex-vivo* studies. However, one of the main problems of cationic liposomes is their lack of stability in serum/plasma (51,52), limiting their use for *in vivo* delivery. Nevertheless, newer formulations such as GS 2888 cytofectin have been developed, which are stable in diluted (10-50%) serum (52).

4.3.1.3. Others

Several other delivery systems are also available: 1) Targeting ASO-containing liposomes coated with maleylated BSA (bovine serum albumin) to macrophages by way of scavenger receptor-mediated delivery (53). 2) Polycationic particles such as polylysine (pLK) and polyethylenimine (PEI) have been successively used for *in vitro* delivery of ASOs. pLK can be conjugated to a specific ligand to target a specific membrane receptor (54), and PEI also have been reported to be very efficient delivery vehicles for ASOs (55,56). 3) Macrophages possess mannose-specific membrane receptors, which recognize and internalize glycoproteins bearing mannose residues (57). Using this principle, Liang et al (58) prepared a molecular complex of mannosylated pLK and ASO. Using such a preparation, in alveolar macrophages, they observed a 17-fold increase in the uptake of an ASO as compared to 'naked' oligonucleotide delivery. For additional delivery vectors please refer to the review by Garcia-Chaumont (37).

4.3.2. *In vivo* delivery of ASOs

4.3.2.1. 'Naked' ASOs

The choice of the *in vivo* delivery system depends upon the type of the cells or organs targeted. Studies on the *in vivo* delivery of ASOs using adjuvants are rather limited because in many cases, ASOs have been successfully delivered with 'naked' ASOs alone. In most of these studies, 'naked' ASOs were directed against genes involved in tumor growth, cardiovascular diseases, inflammatory conditions or infectious diseases (59-66). As mentioned earlier, although many types of ASOs have been designed, the phosphorothioate ASOs are emerging as the compounds of choice for *in vivo* studies. The pharmacokinetic property of the ASO depends upon the size, chemical composition and sequence. Phosphorothioate-ASOs also have high affinity for some proteins. An affinity for serum proteins can affect the delivery process. Most of the injected P-ASOs often accumulate in liver, kidney, muscle and skin (67). In the liver, endothelial cells (56%) rather than Kupffer cells (4%) are the primary targets of 'naked' ASO administration (68). The possible mechanisms by which 'naked' oligonucleotides enter the cells have been reviewed (69). Internalization of ASOs by pinocytosis and also following adsorption onto cell surface proteins such as heparin-binding proteins, have been suggested. Nevertheless, very little of the internalized ASOs were expected to reach the nucleus suggesting that most of the internalized ASOs were trapped in the endosomal/lysosomal compartment. Any release of the ASO into the cytosol would be only due to accidental rupture of the endosomal vesicles. Consequently, relatively high extracellular concentrations (compared to facilitated delivery) of the ASOs would be necessary for efficacy. With regard to Kupffer cells/macrophages, as will be discussed later in detail, 'naked' delivery is not the most efficient delivery system for ASOs. In order to circumvent the endosomal/lysosomal barrier, other delivery systems including liposomal and non-liposomal systems have been developed.

4.3.2.2. Liposomal delivery systems

4.3.2.2.1. Anionic liposomes

One of the problems (but at times an advantage) of using liposomes in gene therapy is that, they are easily eliminated by the RES. Thus, liposomes are the preferred delivery systems for macrophages/Kupffer cells since they represent the RES. The challenge however, is to design an ideal liposomal preparation that is stable in plasma, efficiently sequestered by the Kupffer cells and more importantly, the contents delivered to the cytoplasm/nucleus past the endosomal/lysosomal barrier. Earlier, we reported on an efficient sequestration (>50%) of oligonucleotide-encapsulated anionic liposomes by liver (Kupffer cells) and spleen (splenic macrophages) (70). More recently, we also reported that as opposed to pH-insensitive liposomes, only pH-sensitive liposomes showed efficacy (50-70%) when ASO against TNF-alpha mRNA was targeted (46).

4.3.2.2.2. Cationic liposomes and immunoliposomes

Although new formulations of cationic lipids that are stable in serum are emerging, currently there are no reported studies on nontoxic formulations that can be used for *in vivo* delivery of ASOs for Kupffer cells or macrophages (37). Furthermore, the charge ratio (positive vs. negative) between cationic lipid and ASO plays a critical role in biodistribution of the ASOs (71,72). Thus, there are several complications in using cationic liposomes

for *in vivo* delivery of ASOs. However, there seems to be more promise for immunoliposomes. Alino et al (73) using ASO-encapsulated liposomes covalently coupled with streptavidin on the surface and bound to biotinylated anti-CD45 antibodies showed increased survival of SCID mice after tumor implant. Such approaches of selective targeting could prove useful in targeting Kupffer cells as well.

5. TARGETING KUPFFER CELLS WITH ASOs

5.1. *Ex vivo* studies

As it is always the case, before testing the in vivo efficacy of any antisense oligonucleotide, the efficacy of several ASO constructs are tested in primary cultures of Kupffer cells or in macrophage-like cell lines. In Kupffer cells, in an attempt to control the LPS-induced production of TNF-alpha, Tu et al (21) carried out a systematic study to obtain an effective ASO against this proinflammatory cytokine. In spite of the fact that there are limited studies on the effects of ASOs in primary cultures of Kupffer cells, their studies (21) have provided valuable insight into the selection of ASOs for future therapeutic use. As indicated earlier, after testing the efficacy of various ASO constructs, they came to the conclusion that ASOs containing the TCCC motif (complementary to GGGA motif on the TNF-alpha RNA primary transcript) were the most effective in inhibiting the LPS-induced production of TNF-alpha. Further, when various fragments of the primary transcript containing the GGGA motif were targeted, the most effective ASO (TJU-2755) was the one targeted to the 3'-untranslated region. Otherwise, the ASOs were most effective in the intron regions, suggesting that the ASOs were acting in the nucleus, most likely by RNAse-mediated cleavage (24). In all these studies, ASOs were delivered by cationic liposomes. The ASO TJU-2755, inhibited the LPS-induced production of TNF-alpha by >90% and a comparable reduction in TNF-alpha mRNA. By contrast, neither the scrambled (TJU-2755RD) nor the sense (TJU-2755ss) strands had any effect. The in vivo efficacy of TJU-2755 is discussed in the next section.

In other macrophage cell types such as alveolar macrophages, Liang et al (58) have shown that ASOs can be targeted using mannosylated poly(L-lysine). This approach utilizes the presence of mannose receptors on alveolar macrophages. Although the ASO was efficiently taken up by the cells, no efficacy studies were carried out in that report. In another study, Chaudhuri (53) demonstrated that ASOs encapsulated in liposomes coated with maleylated bovine serum albumin (MBSA) were highly effective against the parasite Leishmania mexina amazonesis, which had infected cultured murine macrophages. In that study, the specific affinity of MBSA for scavenger receptors present on the macrophages was exploited.

5.2. *In vivo* studies

As mentioned earlier, the ASOs and the vectors have to overcome a multitude of challenges from blood-borne components even before they are presented to the target cells. Once sequestered by the cells, the second challenge is to overcome the endosomal/lysosomal barrier. Taking these issues into consideration, several delivery systems have been attempted to target Kupffer cells.

5.2.1. 'Naked' ASOs

One of the most commonly used modes of ASO delivery has been to use P-ASOs without any adjuvants, 'naked'. Although infrequently used to target Kupffer cells, the pharmacokinetic studies of 'naked' ASO administration provide useful information on the uptake of ASOs by Kupffer cells. Generally, when ASOs are administered intravenously, they are extracted from circulation almost completely within one-two hours (74,75). A significant portion (10-20%) of the injected ASO appears in the urine within 24 h. Liver, kidney, skin and muscle tissues are some of the major organs which take up ASOs (67,74). In the liver, ASOs are sequestered by scavenger receptors and are preferentially taken up by endothelial cells (56% vs 40% by parenchymal cells and 4% Kupffer cells) (68). A more recent study by Biessen et al (76) using various types of phosphodiester ASOs showed that the half-life of 'naked' oligos in bloodstream was of the order of 0.6-0.7 min in rats. They also observed that liver and bone marrow were the major targets and that the G (guanine nucleotide) content of the ASO influenced hepatic uptake; higher the G content, lower the uptake by the liver. Thus, there are several factors which affect the uptake of ASOs by the liver and more importantly, Kupffer cells are not the target cells of choice.

5.2.2. Modified ASOs.

It is obvious from the above that improved methods of delivery have to be developed to more specifically target Kupffer cells *in vivo*. In a recent study, Bijsterbosch et al (77) showed that a complex, made of cholesteryl-conjugated P-ASOs and lactosylated low-density lipoprotein (LDL), was efficiently (72%) taken up by the liver within 60 min of intravenous injection. Further, among the liver cells, Kupffer cells accounted for 44% of liver-associated ASO and parenchymal and endothelial cells had 17% and 39% respectively. By contrast, under similar conditions, when two separate ASOs were injected without adjuvants, the extent of Kupffer cell uptake ranged from 8-15%. In their study, the apoprotein of the ASO-LDL complex, was derivatized with galactose residues to enhance specificity of uptake by Kupffer cells. Uptake of lactosylated LDL by Kupffer cells is mediated by galactose-particle receptors, which are different from the classical asialoglycoprotein receptor and are expressed only on Kupffer cells (78). However, there was no report on the in vivo efficacy of these ASOs and hence, it is not clear whether the sequestered ASOs remained entrapped in the vesicular compartment or were released into the cytosol (77). In the delivery of ASOs, it should be taken into consideration that unlike a smaller molecular weight compound such as cholesterol, it would be much more difficult for a larger molecule such as an ASO (MW 5.5-6.5KD) to passively leak out of the lysosomal membrane. Nevertheless, the data show that cholesteryl-conjugated P-ASOs complexed with lactosylated low-density lipoprotein could have a potential in the delivery of ASOs to Kupffer cells.

5.2.3. Liposomal delivery of ASOs.

5.2.3.1. Anionic liposomes

Kupffer cells, being part of the RES, recognize an array of negative charge and hence can efficiently sequester particulate material such as anionic liposomes. Liposomes,

prepared from naturally occurring lipids are unlikely to elicit immune response. Further, anionic liposomes have been approved by FDA, and thus, are an attractive delivery vehicle for *in vivo* delivery of ASOs to Kupffer cells. Studies on the *in vivo* delivery of ASOs were initiated in our laboratory using anionic liposomes containing phosphatidyl choline (PC), phosphatidyl glycerol (PG) and cholesterol (70). Factors taken into consideration were; size, encapsulation efficiency, shelf-life and efficiency in targeting Kupffer cells. The liposomal size ranged from 0.7-2.0 μm, a size a far above the size of the fenestrations in the sinusoids of the liver, and hence, prevented them from crossing the endothelial barrier. A reasonably high (20-40%) encapsulation is obtained when liposomes were prepared by the reverse phase method (70). The oligonucleotide-encapsulated liposomes are stable (90%) for at least 2 weeks when stored at 0-4^{0}C. When these liposomes are intravenously injected, at 90 min post injection, at least 40% of the injected dose (1.2 mg/Kg body weight) of ASO is found in the liver and 10% in the spleen in the intact form (70). The extent of incorporation into lungs and kidneys is less than 5% and is undetectable in all other organs including muscle, brain, testes and intestines. Further, greater than 65% of the liver-associated ASO is found in Kupffer cells. It was calculated that for an injected dose of 1.2 mg of ASO per Kg body weight, the intracellular concentration of the ASO in the Kupffer cells would be about 65 μM. Despite an efficient targeting of Kupffer cells by these anionic liposomes, subsequent *in vivo* efficacy studies showed that an ASO TJU-2755, against TNF-alpha mRNA (21), delivered using these liposomes were ineffective against LPS-induced production of TNF-alpha (46). It was concluded that the sequestered liposomes, most likely, remained trapped inside the endosomal/lysosomal compartment without releasing the ASOs into the cytosol, or that the ASOs released within the lysosomal compartment were degraded by the hydrolytic enzymes. It was therefore, necessary to develop anionic liposomal formulations that would destabilize the endosomal/lysosomal membrane barrier such that the ASOs would escape out into the cytosol. Nevertheless, the studies described above demonstrated that anionic liposomes are effectively sequestered by Kupffer cells and splenic macrophages.

5.2.3.2. Anionic liposomes: pH-sensitive

In macrophages, during phagocytosis, the pH of the endosomal vesicles drops steadily; it drops to about pH 6 within five minutes (44). The pH continues to drop and the intracellular processing of the endosomes continues with the fusion of lysosomes. It is well known that the lysosomal enzymes are optimally active in an acidic environment in the pH range of 4-5. Unlike PC-containing liposomes which are insensitive to acidic pH (41,42), liposomes made of PE are unstable and fusogenic in the acidic environment (41). Earlier studies (41,43,44,79) suggest that upon endocytosis, and acidification by a proton pump in the membrane, the pH-sensitive liposomes fuse with the endosomal membrane and destabilize the endosomal compartment, resulting in the release of the contents into the cytosol.

In a recent study (46), we reported the *in vivo* efficacy of P-ASO TJU-2755 against LPS-induced production of TNF-alpha, in which, the ASO was delivered following encapsulation in pH-sensitive liposomes. The liposomes were prepared using a mixture of PE, CHEMs and cholesterol as described in detail by Ponnappa et al (46). Addition of cholesterol to the mixture provided stability to the preparation; the encapsulation efficiency ranged from 16-20% and had a shelf-life (at 0-4^{0}C) of at least 4 weeks at pH 7.4. The liposomes were formulated to destabilize below pH 6 such that, upon endocytosis by Kupffer cells, they would destabilize the endosomal membrane much before the lysosomal enzymes would be optimally active. The liposomes, at concentrations of the lipid (0.4-0.5 mg/ml) that were likely to prevail in circulation following intravenous injection, were quite stable, as indicated by >80% stability in plasma (46). In male Sprague-Dawley rats, *in vivo* delivery of TJU-2755 in these liposomal preparations showed efficacy at 48 h post intravenous injection. At 48 h following two daily doses (1-2 mg/Kg body wt.) of ASO TJU-2755, the level of undegraded ASO in the liver was about 15 μg/g tissue corresponding to an approximated intracellular concentration of 70-80 μM in Kupffer cells (70) Under those conditions, the LPS-induced production of TNF-alpha in the liver was reduced by 50%. More strikingly, under similar conditions, the plasma TNF-alpha level was reduced by 70% compared to preparations from animals injected with "empty" liposomes. Interestingly, in another parallel study (Tu et al, unpublished observations), it was observed that when rats were intravenously injected with two daily doses of a much higher concentration (10 mg/Kg body weight) of 'naked' TJU-2755, LPS-induced production of TNF-alpha in the liver was also reduced by 50%, with no significant change in the plasma levels of the cytokine. In that context, it should also be pointed out that unlike liposomally delivered ASOs (46), 'naked' ASOs poorly target the spleen. Studies by Rifai et al (75) as well as data from our laboratory (unpublished observations) have shown that less than 1% of the 'naked' ASOs are taken up by the spleen, suggesting that splenic macrophages may also play a role in the LPS-induced TNF-alpha homeostasis. In support of that concept, it was observed that splenectomy prevented LPS-induced liver damage (80). However, further studies are needed to clearly establish the role of other organs of the RE system such as spleen (splenic macrophages?) in LPS-induced liver damage.

In recently concluded studies (81), the *in vivo* efficacy of TJU-2755 was assessed against LPS-induced liver damage in ethanol-fed rats. Liver damage was induced in male Lewis rats fed an ethanol-containing liquid diet for 8-10 weeks followed by an intravenous injection of LPS (2 mg/Kg body wt.). Pretreatment of the animals with two daily doses (2 mg/Kg body wt.) of TJU-2755 in pH-sensitive anionic liposomes prevented liver damage by 60-70%, as assessed by the release of liver enzymes and histology. These results show that using pH-sensitive anionic liposomes as the delivery vehicle, ASO TJU-2755 can be used effectively against TNF-alpha-mediated liver injury. The liposomal preparation was stable in minimally diluted plasma (90%) and had a shelf-life of more than 4 weeks, properties that potentially qualify these preparations for future therapeutic use.

6. PERSPECTIVE

During the past several years, antisense oligonucleotides have emerged as a new generation drugs, with great therapeutic potential. The first antisense drug,

Vitravene® (ISIS Corporation, Carlsbad, CA), was recently approved by the FDA and several antisense constructs are currently undergoing phase III clinical trials for a variety of conditions. The field of antisense oligonucleotides is presently concerned with developing partially non-polar antisense oligonucleotides that would be absorbable in the intestines allowing oral administration. Non-polar groups are attached to chimeric oligonucleotides that contain 6-7 bases of phosphorothioate deoxyoligonucleotides (thus eliciting to RNAse H action), which are extended with "wings" of phosphorothioate ribonucleotides which allow non-polar group (e.g. isopropyl group) substitution in 2-hydroxyl position of the ribose. Although *in vivo* studies have demonstrated efficacy for oral delivery for many of these new antisense drugs, targeting the Kupffer cell has not been attempted for these new molecules. Nevertheless, as discussed earlier, Kupffer cells can be effectively targeted using facilitated delivery systems.

A new area will likely merge with the pH-sensitive anionic liposome delivery. Viruses have the ability of destabilizing lipidic membranes under acidic conditions due to their content "fusogenic peptides". These fusogenic peptides, mainly the fusogenic peptide of the influenza virus, hemagglutinin, have been used to aid the release of oligonucleotides and genes from lysosomal membranes (56,82-84). These fusogenic peptides, whether covalently linked to the antisense oligonucleotides or added to the anionic liposomal preparations, should improve the cytosolic and nuclear delivery of antisense oligonucleotides. It may also be possible to enhance targeting of Kupffer cells and other macrophages by incorporating mannose residues during the preparation of the liposomes.

An additional challenge will be to generate antisense oligonucleotides that are more active than those that act by hydrolysis of RNA by RNase H. There are two additional advances that deserve attention. Recent studies have shown that incorporation of a short 2',5'-linked oligoadenylates on the 5' end of the phosphorothioate oligonucleotides renders the mRNA target sensitive to ribonuclease L, which is now being tested in a number of conditions (85). Another recent development is the finding that RNA interference (RNAi) or "gene silencing", previously thought to occur only in plants, is also active in mammalian cells (86). In this system, RNA-RNA hybrids are broken down into multiple oligonucleotides fragments that interfere with mRNA translation. Using this approach, an "oligonucleotide-forming system" would be delivered in the form of a plasmid, containing the gene (e.g. TNF-alpha gene) in the reverse (3'-5') direction. The plasmid preparation would be delivered in a pH-sensitive liposome.

Overall, the horizon for ASOs as a class of drugs of the future looks promising. The critical role of Kupffer cell-derived cytokines and prostanoids in the pathogenesis of liver diseases has been described. ASOs can be potentially used to suppress the overproduction of proinflammatory cytokines and prostanoids. In most clinical trials, ASOs are delivered 'naked' for their ease of formulation. However, for Kupffer cells, 'naked' delivery is the least efficient way to deliver ASOs. The new developments for oligonucleotide delivery to Kupffer cells with pH-sensitive liposomes described here, will be a significant improvement over the existent methodologies.

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Abbreviations: ASO, antisense oligonucleotide; CHEMS, cholesterylhemisuccinate; FDA, Food and Drug Administration; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MBSA, maleylated bovine serum albumin; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PEI, polyethylenimine; pLK, polylysine; RES, reticulo-endothelial system; ROS, Reactive oxygen species; TNF, tumor necrosis factor

Key Words: Antisense, Oligonucleotide, TNF-alpha, cytokine, pH-sensitive, Kupffer Cell, Delivery, Liposome, Inflammation, Liver, Review

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