side effects and preserve pharmacological activity. To avoid the ABC phenomenon, several strategies were proposed. Administration regimen variables such as dose and time schedules were controlled. The ABC phenomenon was successfully suppressed by injections of pegylated liposomes[30] and nanoparticles[28] at high doses and injections of pegylated nanoparticles at two-week intervals.[29] However, these approaches limited the availability of pegylated pharmaceuticals. In addition, it has been reported that the ABC phenomenon appeared differently in different animal species for pegylated liposomes.[19,21] Thus, to apply pegylated liposomes in a clinical setting, it is necessary to thoroughly examine these regimens in humans. As an alternative approach, the physicochemical properties of the pegylated pharmaceuticals, such as PEG densities and sizes, were controlled. In our previous report, nanoparticles with various PEG content were prepared by controlling the blend ratios of PLA and PEG-PLA.[29] Furthermore, the nanoparticles were prepared from various types of block copolymers such as PEG-PLA with different PEG lengths (M_w 2000, 11400, and 24500), PLA-PEG-PLA (A-B-A type block polymer; M_w of PEG: 11600 and 24800), and PEG-PLA with a carboxylic group at one end of a PEG segment. However, these nanoparticles all elicited the ABC phenomenon. Likewise, liposomes with different PEG densities and lengths could not suppress the ABC phenomenon.[22] Nevertheless, small pegylated pharmaceuticals may hold promise for ABC suppression. It was reported that polymeric micelles with diameters of approximately 50 nm elicited the ABC phenomenon but that smaller ones with diameters <30 nm did not.[27] In addition, the ABC phenomenon was not induced in humans upon repeated injection of pegylated interferon.[40] Thus, the size of pegylated formulations may be a determinant for induction of the ABC phenomenon. However, obtaining liposomal formulations with diameters <30 nm is technically difficult on account of the lower physical stability of the liposomes. Furthermore, it is difficult to encapsulate drugs in small solid nanoparticles with high efficiency. Taken together, to improve the availability of colloidal carriers, it is necessary to find another way to suppress the ABC phenomenon.

In the present study, we examined the utility of various hydrophilic polymers (PVP, PAcM, PDMAA, and PVA) as coating polymers to suppress the ABC phenomenon. The nanoparticles coated with PVP, PAcM, and PDMAA showed stealthiness but had a shorter half-life than PEG-coated nanoparticles. This lower extent of stealthiness may be due to physical properties such as the density and thickness of hydrophilic polymers on the nanoparticle surfaces. In our previous report, we showed that the extent of nanoparticle stealthiness increased with the PEG content.[29] Thus, nanoparticles were also prepared from various blended ratios of PLA and PVP-PLA with assorted molecular weights. However, the extent of stealthiness was not further improved. Gaucher et al. reported that PVP-coated nanoparticles exhibited shorter circulation times than PEG-coated nanoparticles,[41] although both nanoparticles had considerably shorter half-lives than the nanoparticles shown in this study, probably due to the lower molecular weights of the hydrophilic polymer segments. In the Gaucher report, it was clarified that protein adsorption patterns on both nanoparticles were distinct both quantitatively and qualitatively.[41] Overall, the limited extent of stealthiness shown in this study may depend on the inherent properties of the polymers.

It is generally believed that because of the EPR effect, the stealth carriers remain in the blood for prolonged periods of time, which this leads to their higher accumulation in lesions such as solid tumors and inflammatory lesions. Although it is unclear whether the PVP-NP included in this study can efficiently accumulate in the target lesions because of the EPR effect, their stealthiness may be sufficient to induce therapeutic activity. Higher accumulation does not necessarily correspond to higher therapeutic activity because therapeutic activity is affected by multiple factors such as the level of accumulation in the lesions, local distribution in the lesion, and the drug release behaviors of the carriers.[42] We observed that the corticosteroid-encapsulating nanoparticles with the longest blood half-life did not have the highest anti-inflammatory activity.[35] In future studies, we plan to determine the utility of PVP-NP using diseased experimental animals and, if required, improve the stealthiness of the nanoparticles accordingly.

Interestingly, PAcM-NP and PDMAA-NP also did not induce the ABC phenomenon (Figure 3), suggesting that PEG is a trigger molecule of the ABC phenomenon. On the basis of the results, we selected PVP as a candidate for post-PEG material, regardless of the similar stealthiness of PAcM-NP and PDMAA-NP, because the safety of PVP has already been well-investigated.[43] PVP has been used as a food additive and a pharmaceutical additive (a tablet-coating aid) in clinical settings.

It was reported that pegylated liposome (Doxil/Caelyx) did not induce the ABC phenomenon because of the immunosuppressive activity of the encapsulated doxorubicin.[23] Indeed, pegylated carriers incorporating immunosuppressive drugs are useful in cases where the carriers need to be administered repeatedly. However, this strategy significantly limits the utility of the carriers and drugs. The development of carriers that do not induce the ABC phenomenon encouraged the use of drugs with low (or no) immunosuppressive activity. It is expected that in addition to cancer, these carriers will be used for different conditions such as inflammation and bloodstream disorders. Furthermore, these carriers may be able to deliver chemically unstable drugs like siRNA. We also evaluated the effect of PGE_1 on the ABC phenomenon because PGE_1 may influence immune responses. When PEG-NPs were used without PGE_1 (empty nanoparticles) for the first dose, the ABC phenomenon was similarly induced. Thus, it was estimated that the effect of PGE_1 was negligible in the range of doses used.

Although the mechanism underlying the ABC phenomenon is still unclear, it is certain that some soluble serum factors produced by preinjection are determinants in this phenomenon.[19,25] As shown in Figure 5, no ABC phenomenon was induced by pretreatment with different types of nanoparticles. This result indicates that the soluble factors produced by the injection of PEG-NP have specificity against PEG. In the case of PVP-NP, it was unclear whether PVP-NP triggered the production of some soluble factors. However, it is certain that PVP-NP produced no soluble factors to accelerate the blood clearance of either PEG-NP or PVP-NP, at least at the doses and the time intervals evaluated here.

IgM antibody is thought to be one of the crucial soluble factors for induction of the ABC phenomenon.[21,26] In our previous report, the level of anti-PEG IgM induced by a single injection of PEG-NP showed an approximate correlation with the extent of the ABC phenomenon.[29] As shown in Figure 6B, PVP-NP did not induce anti-PVP IgM production, which is in accordance with the lack of induction of the ABC phenomenon (Figures 2B and 4B). On the other hand, PEG-NP markedly induced anti-PEG IgM production after a single injection (Figure 6A). However, the levels of production, interestingly, decreased with repeated injections. The ABC phenomenon was induced

7 d after a series of three injections of PEG-NP (50 μg/rat) at 7 d intervals (Figure 4A), but anti-PEG IgM was only slightly produced under that regimen. This result implies that IgM level is not always correlated with ABC phenomenon extent. In addition, nonpegylated nanoparticles (NC-NP or PVP-NP) injected in advance did not accelerate the blood clearance of PEG-NP, although it has been reported that nonpegylated liposomes accelerated the clearance of pegylated liposomes.[22,23] These results suggest that a mechanism other than the production of IgM may also be involved and that this mechanism may depend on colloidal formulation. The ABC phenomenon may also be determined by the protein adsorption pattern, since a difference between PEG and PVP patterns has been shown.[41] Although further study is necessary to clarify these findings, we are certain that the mechanism of the ABC phenomenon is quite complex.

Aside from the question of the mechanism, it is significant that PVP-NP did not induce the ABC phenomenon. This suggests that the colloidal carriers coated with PVP can show the same pharmacokinetics and pharmacodynamics irrespective of the regimen used for administration. Thus, the use of PVP as a post-PEG agent may increase the utility of colloidal drug carriers in the future.

Conclusions

In the present study, we prepared nanoparticles from a mixture of PLA homopolymer and block copolymers consisting of PLA and various hydrophilic polymers. PVP-coated nanoparticles showed prolonged blood residence, although the extent of stealthiness may require improvement. The ABC phenomenon was not induced upon repeated injection of PVP-coated nanoparticles at various time intervals, dosages, or frequencies, while it was elicited by PEG-coated nanoparticles. Thus, PVP may be an interesting polymer for novel therapeutics in the future.

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Supporting Information Available. The structural formula of the polymers and additional ELISA antibody detection data. This material is available free of charge via the Internet at http://pubs.acs.org.

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EXPERT REVIEW

Targeting Anticancer Drugs to Tumor Vasculature Using Cationic Liposomes

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ABSTRACT Liposomal drug delivery systems improve the therapeutic index of chemotherapeutic agents, and the use of cationic liposomes to deliver anticancer drugs to solid tumors has recently been recognized as a promising therapeutic strategy to improve the effectiveness of conventional chemotherapeutics. This review summarizes the selective targeting of cationic liposomes to tumor vasculature, the merits of incorporating the polymer polyethylene-glycol (PEG), and the impact of the molar percent of the cationic lipid included in cationic liposomes on liposomal targeting efficacy. In addition, the discussion herein includes the therapeutic benefit of a dual targeting approach, using PEG-coated cationic liposomes in vascular targeting (of tumor endothelial cells), and tumor targeting (of tumor cells) of anticancer drugs. Cationic liposomes have shown considerable promise in preclinical xenograft models and are poised for clinical development.

KEY WORDS angiogenesis · anti-angiogenic therapy · anticancer drugs · dosing schedule · dual targeting · PEG-coated cationic liposome · vascular targeting

ABBREVIATIONS

5-FU	5-fluorouracil
A-Mel-3	Amelanotic melanoma
AUC	Area under the blood concentration *versus* time curve
bFGF	Basic fibroblast growth factor
CHOL	Cholesterol
DAS model	Dorsal air sac model
DC-6-14	O,O′-ditetradecanoyl-N-(alpha-trimethylammonioacetyl)diethanolamine chloride
DC-Chol	3β-(N-(N′,N′-dimethylaminoethane) carbamoyl) cholesterol
DOPE	Dioleoylphosphatidylethanolamine
DOTAP	Dioleoyl trimethylammonium propane
DXR	Doxorubicin
EPR	Enhanced permeability and retention
HSPC	Hydrogenated soy phosphatidylcholine
K14-HPV16	The oncogene from the human papilloma virus (HPV) is driven by a region of the keratin 14 (K14) promoter
LLC	Lewis lung carcinomas
l-OHP	Oxaliplatin
LS174T	Human epithelial colon cell line
MMPs	Matrix metalloproteinases
$mPEG_{2000}$-DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]
MPS	Mononuclear phagocyte system
NGR	Asn-Gly-Arg
PEG	Polyethylene glycol
RIP-Tag2	Expression of the SV-40 virus large T antigen (Tag)[1] oncogene is driven by the 5′ flanking region of the rat insulin gene including the promoter (RIP).
SCID	Severe combined immunodeficient
VEGF	Vascular endothelial growth factor

INTRODUCTION

Tumor vasculature is the general route of entry by which chemotherapeutic agents gain access to tumor cells. The vasculature also represents the life support of these target cells. Therefore, interrupting the flow of oxygen and nutrients to tumor tissue may create an opportunity to effectively manage tumor growth and the progression of

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disease (1–3). Targeting the tumor vasculature, instead of the tumor cells themselves, is assumed to have several advantages. Normal endothelial cells are quiescent, and, therefore, side effects in the non-target endothelium are expected to be minimal (4). Proliferating endothelial cells in solid tumors share similar phenotypes—even when different solid tumors are compared. This makes vascular targeting applicable to a wide variety of tumor types (5). Furthermore, endothelial cells are genetically stable (unlike tumor cells); thus, there is a reduced risk of developing drug resistance (6). Finally, endothelial cells in tumor vessels are more accessible to circulating chemotherapeutics than tumor cells are, because the vasculature of a tumor occupies a relatively small area in comparison with the tumor interstitium, and most anticancer agents are applied intravenously (7).

Nonetheless, despite the many advantages of the vascular targeting approach, one potential problem is the lack of specificity of free anticancer agents. It is, therefore, a formidable challenge to minimize the total amount of a drug being delivered to healthy tissues while improving selective delivery to tumor targets. The need for an effective way to overcome this problem is obvious. The loading of low-molecular-weight anticancer drugs onto nanomolecular drug delivery systems has been shown to promote selective delivery of anticancer drugs to solid tumors by altering the biodistribution of associated drugs.

Many approaches based on nanocarrier drug delivery systems have been applied to achieve the targeting of anticancer agents to tumor tissue, including vasculature (Fig. 1) (8–10). Cationic liposomes, the focus of this review, are a promising carrier system for the delivery of anticancer agents to tumor endothelial cells, which takes advantage of the natural affinity of cationic molecules at the surface of the carrier system for anionic molecules, such as glycoproteins, anionic phospholipids, and proteoglycans, in the tumor microvasculature.

TUMOR ANGIOGENESIS

Tumor angiogenesis, the formation of neovessels from pre-existing vessels in solid tumors, is critical for the support of tumor growth and progression, not only by providing nutrients, oxygen, growth factors and other substances to tumor cells, but also by allowing metastatic cells into circulation (11,12). Tumors can gain sufficient nutrients and oxygen by simple diffusion up to a size of 1–2 mm, at which point their further growth requires the elaboration of a vascular supply (13). The process of tumor angiogenesis involves recruitment of the neighboring host mature vasculature to begin sprouting new blood vessel capillaries, which grow toward, and subsequently infiltrate, the tumor mass (14). In addition, tumor angiogenesis might involve the recruitment of circulating endothelial precursor cells from the bone marrow to evoke neovascularization (15,16). Tumor angiogenesis is mainly triggered by growth factors in the microenvironment, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and the matrix metalloproteinases (MMPs). These activating factors are produced by the tumors themselves, by the surrounding host tissue, or by infiltrating macrophages and fibroblasts in the tumor (17,18). Most of these activating compounds exert their actions through endothelial cell surface receptors, for which they serve as ligands, leading to secretion of additional angiogenic factors (13). Suppression of the angiogenesis process leads to eradication of primary tumor cells and suppression of metastasis, which makes this a promising strategy for the treatment of solid tumors (anti-angiogenic therapy).

VASCULAR STRUCTURE AND TARGETING

Vascular Structure of Solid Tumors

With the increasing promise of vascular targeting in solid tumors, a thorough understanding of the cellular structure and function of tumor vessels has become even more important. In comparison with blood vessels in normal tissues, tumor vessels are recognized as dynamic, both in terms of the formation of new vessels by angiogenesis and the remodeling of existing vessels (19,20). Tumor vessels are often dilated and irregular in distribution and shape and have abnormal pericytes and basement membrane coverage (21,22). As a result, vascular function is compromised. In addition, tumor blood vessels exhibit endothelial cell gaps, with an average size of ~100–600 nm (23). These pores are significantly larger than the gaps found in normal endothelium, which are typically <6 nm wide (24). This porous nature of tumor vasculature enables the preferential accumulation of macromolecules and polymeric drugs in tumor tissue, via a passive targeting phenomenon known as the enhanced permeability and retention (EPR) effect (25,26).

Tumor vessels are also characterized by the overexpression of specific surface receptors and antigens and by negatively charged macromolecules, such as glycoproteins, anionic phospholipids and proteoglycans (27–29). Such molecules can serve as exploitable selective targets to achieve active vascular targeting of chemotherapeutic agents by means of nanocarrier systems.

Vascular Targeting with Liposomes

As described above, tumor endothelial cells overexpress specific cell surface antigens, which are absent or barely

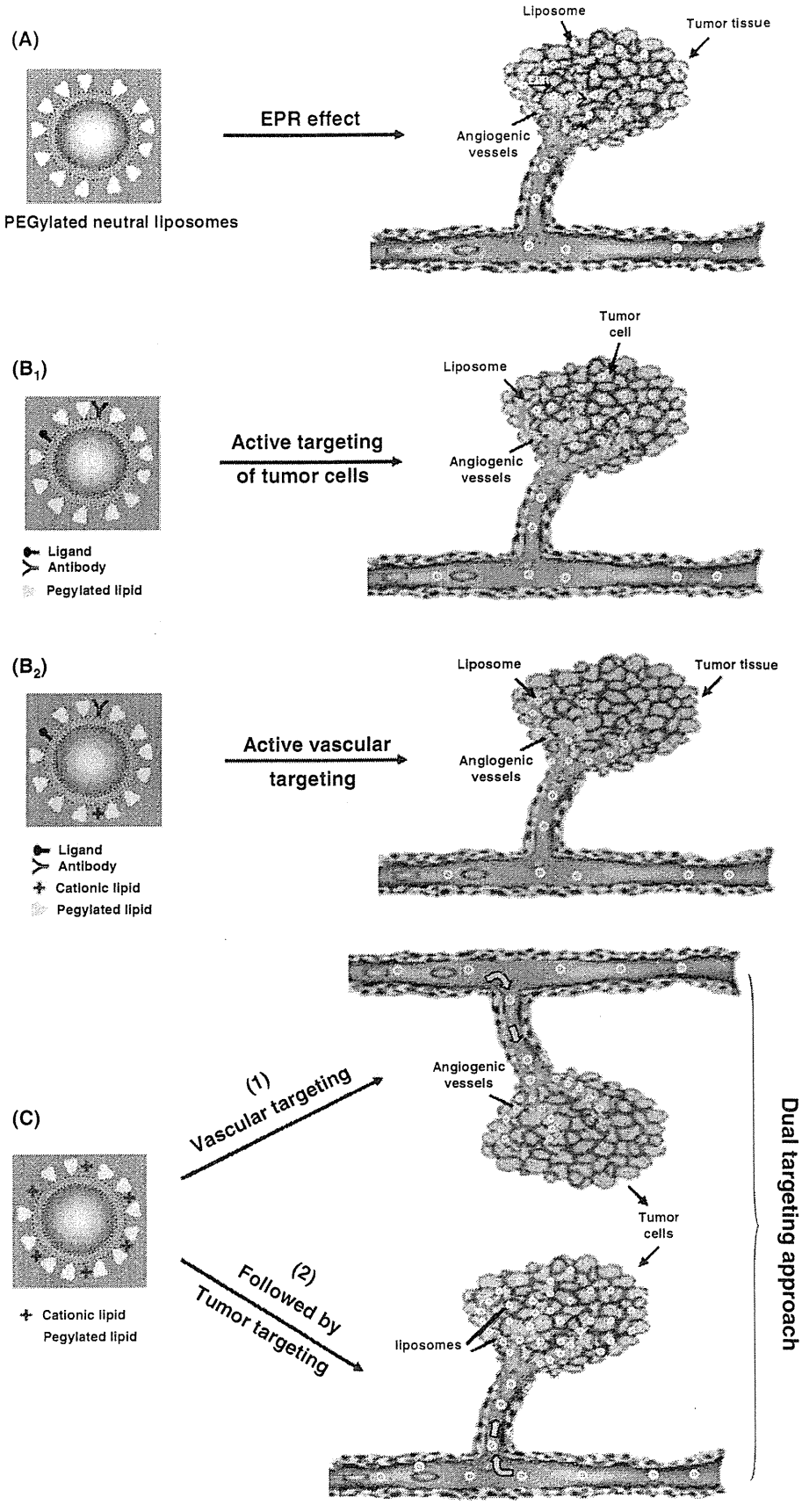

Fig. 1 Cartoon depicting different targeting approaches to tumor vasculature and/or tumor cells with liposomal drug delivery system. (A) Passive targeting of PEGylated liposomes via the leaky vasculature of solid tumor resulting in localization of the liposomes within the tumor interstitial space. (B_1) Active targeting of tumor cells with ligand (antibody or peptide)-targeted liposomes against cell surface antigens expressed selectively on tumor cells. (B_2) Active targeting of tumor vasculature with liposomes modified with ligand (antibodies, peptides or charge) against specific antigens overexpressed exclusively on the surface of tumor endothelial cells. (C) Dual targeting approach (*i.e.* targeting both tumor cells and tumor endothelial cells) with PEG-coated cationic liposomes. In dual targeting approach, PEG-coated cationic liposomes, by virtue of their surface positive charge, bind selectively to tumor endothelial cells via electrostatic interaction. Then, after the saturation of binding sites on the surface of tumor endothelial cells, the liposomes begin to extravasate through the leaky tumor vasculature into the tumor interstitial by EPR effect. Once being inside the tumor interstitium, PEG-coated cationic liposomes bind to tumor cells. Therefore, cytotoxic agents encapsulated in PEG-coated cationic liposomes will be allowed to exert their cytotoxic effect via a dual targeting approach as a result of affecting both tumor endothelial cells and tumor cells.

detectable in normal blood vessels. This unique characteristic of tumor endothelial cells can be exploited to achieve active vascular targeting by means of nanocarrier drug delivery systems, such as liposomes. Many approaches have been applied to enhance the targeting efficiency of liposomes to tumor endothelial cells (8,9,30). They include the coupling of specific molecules, such as antibodies, specific peptides, or growth factors, or incorporating cationic charges on the surface of liposomes. This review, however, is focused on cationic liposomes as one of the most promising carriers for targeting tumor vasculature.

CATIONIC LIPOSOMES

Nearly two decades have passed since the introduction of cationic liposomes in gene therapy (31). Cationic liposomes have proved to be an effective tool for gene delivery, both *in vitro* and *in vivo* (32–34). They have several advantages over viruses as gene transfer vectors. Unlike viral vectors, they can be used to transfer DNA of essentially unlimited size. In addition, they are technically simple and quick to formulate, have low immunogenicity, and are readily available commercially (35). There are, however, some drawbacks with lipid vectors, including lower efficiency than viral vectors in gene transfer, and transient gene expression (36–38). Such drawbacks prompted a re-evaluation of their use as gene vectors.

Recently, there has been renewed interest in cationic liposomes, mainly due to their inherent, yet unexplained, ability to selectively target tumor vasculature. This selective affinity of cationic liposomes to tumor vasculature provides an opportunity for the development of many anti-angiogenic and/or anticancer formulations based on cationic liposomes. In addition, a wide area of research has focused on manipulating the structural features of cationic liposomes to improve their vascular targeting efficiency and reduce toxicity-related reactions.

In Vivo Toxicity of Cationic Liposomes

Cationic liposomes possess many physical characteristics that make them attractive as candidates for gene/anticancer drug delivery to solid tumors. However, a major limitation is their toxicity. It is assumed that their toxicity depends mainly on the type of cationic lipid incorporated in their composition. For example, biodegradable ester lipids are less toxic in cell culture than ether lipids, which are more resistant to enzymatic hydrolysis (39,40). Lipids that do not form micelles, such as double-chain lipids, are less toxic in cell culture than single-chain cationic surfactants, which do adopt a micellar structure (41). When administered to animals, cationic liposomes tend to aggregate with serum proteins or blood cells (42) and adhere electrostatically to vascular endothelium cells after intravenous injection, resulting in their rapid clearance from the bloodstream or their localization in the lungs with the risk of causing lung embolisms (43).

In addition, inflammatory reactions have been observed upon *in vivo* use of cationic liposomes. However, the mechanism by which cationic liposomes elicit such inflammation is still not completely understood. Freimark *et al.* (44) reported that intratracheal instillation of cationic liposomes induced cytokine production and cellular influx in the lung airways, leading to severe lung inflammation. Dokka *et al.* (45) demonstrated that instillation of cationic liposomes elicited dose-dependent toxicity and pulmonary inflammation. They found that the inflammatory reaction was correlated with an oxidative burst that resulted from a dose-dependent increase in the generation of reactive oxygen intermediates induced by cationic liposome instillation.

Immune responses to cationic lipids that are frequently used to formulate cationic liposomes are much more enigmatic. However, Zelphati *et al.* (46) have shown that the serum complement system can be activated by cationic lipids incorporated in the liposomal membrane. Complement activation could result in complement components binding to the liposomes, thereby leading the liposomes to be recognized by the receptors for complement components found in lungs or liver (47). This could also explain the rapid clearance of cationic liposomes from the blood and enhanced uptake by lungs or liver. These studies made the assumption that PEGylation is necessary for *in vivo* use to protect cationic liposomes against immune reactions, aggregation with blood components, and enhanced uptake by the lungs and/or a mononuclear phagocyte system (MPS).

Inclusion of PEG in Cationic Liposomes

As described above, cationic liposomes have attracted attention as potential carriers to deliver therapeutic genes (48–50) and cytotoxic drugs specifically to the tumor vasculature (neovascular therapy) (51–53). A potential limitation, however, is the propensity of cationic liposomes to be rapidly eliminated from circulation by the "first-pass" organs, such as the lungs, the liver and the spleen (54). The inclusion of high-molecular-weight polymers, such as polyethylene glycol (PEG), in the liposome surface is considered an efficient approach to limit the interaction of conventional liposomes with circulating blood proteins, blood cells or cells of MPS, and thus to prolong their blood circulation time (55,56). The mechanism for the protective effect imparted by the inclusion of PEG at the surface of the liposome is believed to be the formation of a physical barrier, a hydration zone, around the liposomes, related to the hydrophilic nature of the grafted PEG at the

surface of the liposomes. This zone of exclusion diminishes liposome-protein interaction as a result of long PEG polymer chain constrictions (57,58).

Many studies have focused on elucidating the optimal concentration of PEG that should be incorporated in the liposomal membrane to provide liposomes with long-circulating characteristics. Levchenko *et al.* (59) demonstrated that PEG concentrations of ≥6 mol% shield the electric surface potential of cationic liposomes, while higher concentrations (≥15 mol%) were found to cause unfavorable structural changes in the liposomal bilayer, and thus enhance the rapid clearance of liposomes from circulation. For this reason, approximately 5–10 mol% of PEG-lipid derivative is included in the preparation of PEG-coated cationic liposomes. It should be noted that the partial coating of cationic liposomes with PEG does not alter their affinity to bind to tumor vascular surfaces (60). Campbell *et al.* (61) reported that although the zeta potential of cationic liposomes coated with 5 mol% of PEG was lower than that of their uncoated counterparts, PEG-coated cationic liposomes still retained the ability to associate with tumor endothelial cells. Furthermore, the inclusion of PEG-lipid derivatives on the liposome surface imparts long-circulating characteristics and, thus, enhances the therapeutic efficiency of the encapsulated drugs.

Effect of Surface Charge of Cationic Liposomes on Tumor Vascular Targeting

The targeting efficiency of cationic liposomes to tumor endothelial cells is strongly governed by the degree of surface cationic charge. Krasnici *et al.* (62) demonstrated that intravenously applied cationic liposomes, but not anionic or neutral liposomes, preferentially accumulate in the amelanotic (A-Mel-3) melanoma of the hamster *in vivo*, in comparison with normal surrounding host tissue. This preferential accumulation of cationic liposomes in the solid tumor was caused mainly by binding of the liposomes to angiogenic tumor microvessels, whereas neutral and anionic liposomes extravasated passively into the parenchyma. Campbell *et al.* (61) showed that an increase in cationic lipid from 10 to 50 mol% in PEG-coated cationic liposomes led to a 2-fold increase in liposomal accumulation in tumor vessels.

Besides binding to angiogenic endothelial cells, intratumoral clearance of liposomes is also dependent on the liposomal surface charge. Karsnici *et al.* (62) demonstrated that, in a tumor-bearing mouse model, anionic liposomes were almost cleared from the tumor, as well as the tumor tissue, at 3 h after intravenous injection, while cationic liposomes were retained within the tumor vasculature for up to 6 h after injection. Nomura *et al.* (63) reported that, in tissue-isolated tumor perfusion systems, clearance of positively charged liposomes was greatly retarded in comparison to neutral liposomes, which immediately appeared in the venous outflow perfusate following intra-tumoral injection. Recently, using a tumor-bearing mouse model, Abu Lila *et al.* (64) emphasized that PEG-coated cationic liposomes showed 2–3-fold higher accumulation in tumor tissue than PEG-coated neutral liposomes. It is interesting that tumor accumulation of PEG-coated cationic liposomes tended to increase up to 24 h after injection and remained at this level at 48 h after injection. Tumor accumulation of PEG-coated neutral liposomes, on the other hand, was substantially lower, and reached a maximum at 24 h post-injection followed by a gradual decline during the next 24 h. They ascribed this enhanced intra-tumor accumulation of PEG-coated cationic liposomes to the selective binding of PEG-coated cationic liposomes—not only to tumor angiogenic vessels, but to tumor cells as well. The preferential and prolonged accumulation of cationic liposomes in angiogenic tumor vessels, therefore, seems to be a general feature of cationic liposomes, and is independent of tumor type.

Pharmacokinetics of Cationic Liposomes

Although many publications discuss the *in vivo* fate of neutral and negatively charged liposomes, surprisingly little is known about the *in vivo* fate of cationic liposomes. Only a few reports about the pharmacokinetics of cationic liposomes exist, and their results are ambiguous or conflicting (65). Generally, cationic liposomes have been viewed as incompatible *in vivo*, giving accelerated clearance from blood circulation (66,67). Litzinger *et al.* (68) studied the biodistribution pattern of cationic liposomes consisting of 3β-(N-(N′,N′-dimethylaminoethane) carbamoyl) cholesterol (DC-Chol)/dioleoylphosphatidyl ethanolamine (DOPE) (1:4, molar ratio). They reported that the injected liposomes accumulated primarily in the liver. More than 60% of the injected dose had accumulated in the liver at 5 min post-injection, while only ~18% of the injected dose remained in blood circulation. Comparatively little accumulation occurred in the lungs (less than 10% of the injected dose). They postulated that the interaction of cationic liposomes with plasma components, including opsonin(s), immediately following injection, may have hindered non-specific binding with cell membranes and accumulation within tissues. This would explain the relatively low accumulation of cationic liposomes in the lungs, the first capillary bed encountered following tail vein injection, and enhanced accumulation in the liver, presumably due to enhanced uptake by Kupffer cells in the liver. In contrast, Ishiwata *et al.* (69) studied the *in vivo* fate of cationic liposomes composed of O,O′-ditetradecanoyl-N-(alpha-trimethylammonioacetyl) diethanol amine chloride (DC-6-14)/DOPE/cholesterol (CHOL)

(4:3:3, molar ratio). A dramatic and transient accumulation of liposomes in the lungs was observed. At 3 min post-injection, ~60% of the injected dose was present in the lungs. The accumulated liposomes were then slowly released from the lungs by the flow of blood and were immediately removed from circulation and accumulated in the liver. This may account for the inverse pharmacokinetic relationship between accumulation in the lungs and the liver.

Some studies have focused on the effect of the molar percent of cationic lipid incorporated into liposomes and the presence of PEG on the *in vivo* fate of cationic liposomes (69,70). Generally, PEG-coated cationic liposomes showed enhanced pharmacokinetic profiles, with longer circulation half-lives than cationic liposomes without PEGylation, as observed in PEG-coated neutral liposomes. In addition, incorporating a high mol% of cationic lipid in the liposomal composition triggers rapid clearance of the liposomes from blood circulation, even though they were coated with PEG. Stuart *et al.* (70) demonstrated that in the absence of PEGylation [1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-n-[methoxy (polyethylene glycol)-2000 [mPEG$_{2000}$-DSPE]]], cationic liposomes consisting of 5–50 mol% cationic lipid, dioleoyl trimethylammonium propane (DOTAP), were rapidly cleared from circulation, resulting in <5% of liposomes present in blood at 24 h post-injection. In addition, PEGylation did not increase the blood levels of liposomes containing 50 mol% cationic lipid (DOTAP/hydrogenated soy phosphatidylcholine (HSPC) in a molar ratio of 1:1). However, when the cationic lipid content was reduced to 20 mol%, inclusion of 5 mol% mPEG$_{2000}$-DSPE significantly increased blood levels of the liposomes. At ≤10 mol% cationic lipid, the inclusion of 5 mol% of mPEG$_{2000}$-DSPE was found to exert a maximal protective

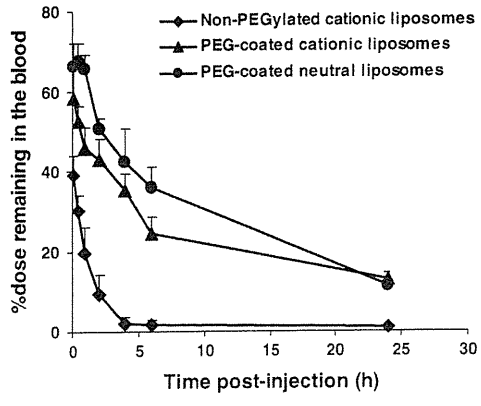

Fig. 2 Blood clearance of various liposomal formulations. On day 12 after tumor inoculation, LLCC tumor-bearing mice ($n = 4$) were injected intravenously with either radio-labeled non-PEGylated cationic liposomes (*filled diamond*), PEG-coated cationic (*filled triangle*) or PEG-coated neutral liposomes (*filled circle*). At selected time points (0.083, 0.5, 1, 2, 4, 6, and 24 h), blood was collected and analyzed for radioactivity.

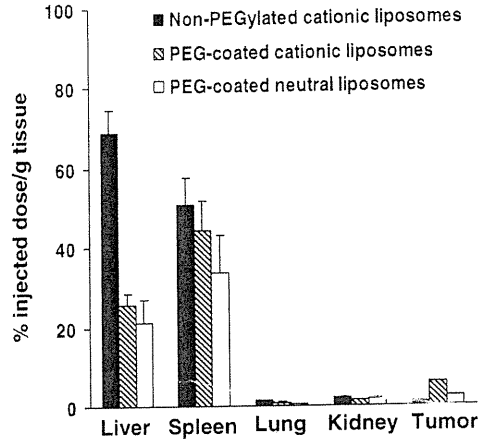

Fig. 3 Organ distribution of different liposomal formulations. On day 12 after tumor inoculation, LLCC tumor-bearing mice ($n = 4$) were injected intravenously with either radio-labeled non-PEGylated cationic liposomes (*closed columns*), PEG-coated cationic (*hatched columns*) or PEG-coated neutral liposomes (*open columns*). At 24 h post-injection, the animals were euthanized and the radioactivity in each organ was determined. Data are presented as mean±SD. (In the case of the spleen, the value was per 500 mg instead of per gram).

effect against the rapid clearance of cationic liposomes from blood circulation. Abu Lila *et al.* (71) also recently confirmed that, in tumor-bearing mice, PEG-coated cationic liposomes consisting of HSPC/CHOL/DC-6-14/mPEG$_{2000}$-DSPE (2:1:0.2:0.2 molar ratio) showed more enhanced pharmacokinetic profiles than non-PEGylated cationic liposomes (Figs. 2 and 3). In addition, PEGylation significantly delayed the rapid clearance of cationic liposomes from blood circulation to an extent similar to that of PEG-coated neutral liposomes consisting of HSPC/CHOL/mPEG$_{2000}$-DSPE (2:1:0.2 molar ratio). However, PEG-coated cationic liposomes showed a lower area under the blood concentration *versus* time curve (AUC) compared to PEG-coated neutral liposomes. It is likely that the lower AUC was a result of the relatively enhanced blood clearance and tissue distribution of PEG-coated cationic liposomes (Table I), due to the presence of a positive charge on the liposomal surface. Interestingly, PEG-coated cationic liposomes and PEG-coated neutral liposomes accumulated in liver and spleen to similar extents (Fig. 3). This demonstrates that PEGylation of the cationic liposomes effectively prevents the rapid clearance of cationic liposomes from the blood circulation as is observed for non-PEGylated cationic liposomes (Fig. 2).

Selective Delivery of Cationic Liposomes to Tumor Vasculature

It is well known that cationic liposomes quite selectively target the vasculature of tumors, a phenomenon not noted with anionic (negatively charged) or electroneutral (zero

Table I Pharmacokinetic Parameters of PEG-Coated Liposomes in Tumor-Bearing Mice

Liposome	Half-life (h)	Clearance (ml/h)	$AUC_{t=0\to\infty}$ (%dose·h/ml)	V_d (ml)
PEG-coated cationic liposomes	10.4 ± 0.7^{ns}	$0.133 \pm 0.007^{*}$	$750.6 \pm 35.2^{*}$	$1.99 \pm 0.10^{**}$
PEG-coated neutral liposomes	9.4 ± 0.5	0.113 ± 0.005	884.9 ± 41.7	1.53 ± 0.07

LLCC tumor-bearing mice received a single intravenous injection of either radio-labeled PEG-coated cationic liposomes or radio-labeled PEG-coated neutral liposomes. Pharmacokinetic variables were determined using PK Analyst software. Data represent the mean±SD ($n = 4$ mice per time point). ns > 0.05, $^{*}p < 0.05$, $^{**}p < 0.01$ against PEG-coated neutral liposomes.

charge potential) liposomes. Campbell *et al.* (61) demonstrated that after the intravenous application of PEG-coated anionic and neutral liposomes in LS174T-bearing mice, there was no selective accumulation in the tumor vasculature. By contrast, PEG-coated cationic liposomes accumulated extensively in tumor vessels. They assumed that the electrostatic interaction between the positively charged surface of cationic liposomes and the negatively charged glycoprotein layer of the tumor endothelium was partly responsible for this preferential accumulation in tumor vasculature. In addition, they proposed that the sluggish and stunted blood flow in tumor vessels, in contrast to the normal continuous flow in most healthy tissues, enhances the interaction between the anionic sites on the dynamic vasculature of the tumor and the cationic liposomes. Chang *et al.* (72) demonstrated that in the case of mosaic tumor vessels, vessels comprised of both vascular endothelial cells and tumor cells themselves, the tumor cells may come in direct contact with cationic liposomes, and uptake may occur in both vascular endothelial cells and tumor cells.

Thurston *et al.* (73) previously demonstrated that, in the RIP-Tag2 and K14-HPV16 tumor models, the amount of cationic liposomes accumulated in tumor vasculature was up to 33-fold higher than that accumulated in vessels in normal tissue of non-tumor-bearing (normal) mice. Recently, Abu Lila *et al.* (51) developed a PEG-coated cationic liposome composed of HSPC/CHOL/DC-6-14/$mPEG_{2000}$-DSPE (2:1:0.2:0.2 molar ratio) and confirmed that, in the dorsal air sac (DAS) model, the PEG-coated cationic liposomes accumulated preferentially and selectively in tumor angiogenic vessels induced in mouse skin. In addition, no selective accumulation/binding to pre-existing blood vessels in the skin was observed. Collectively, these studies provide many lines of evidence that cationic liposomes have the inherent potential to bind selectively to tumor vascular endothelial cells, which may be exploited to achieve successful anti-angiogenic therapy.

Selective Delivery of Anticancer Drugs to Tumors and Their Vasculature

The idea of exploiting accessible anionic sites, along with tumor vessels, by means of PEG-coated cationic liposomes containing anticancer drugs is promising for cancer therapy. Many preclinical studies (see Table II) have addressed the utilization of cationic liposomes to selectively deliver anticancer drugs to tumor vasculature. Some of the studies that illustrate the vascular targeting of anticancer drugs using a cationic liposome-based drug delivery system are described below.

Sengupta *et al.* (74) studied the therapeutic efficacy of etoposide encapsulated in cationic liposomes using a murine fibrosarcoma model. Liposomal etoposide significantly delayed tumor growth when compared with non-liposomal etoposide. In addition, *in vivo* survival studies demonstrated a significant increase in the lifespan of mice treated with etoposide-containing cationic liposomes, compared to mice treated with free (non-liposomal) etoposide. However, the exact mechanism of this enhanced antitumor activity was not completely elucidated in this study. Kunstfeld and co-workers (75) demonstrated that paclitaxel encapsulated in cationic liposomes potentially suppresses tumor angiogenesis and inhibits orthotopic melanoma growth in SCID mice. By contrast, free paclitaxel, while showing an inhibitory effect in *in vitro* cell culture, was unable to significantly suppress angiogenesis and tumor growth *in vivo*. Strieth *et al.* (76) evaluated the therapeutic efficacy of cationic liposomes containing paclitaxel (EndoTAG™-1) in Meth A sarcoma-bearing mice. Drug-containing EndoTAG™-1 resulted in a significant suppression of tumor growth, compared to free paclitaxel. The authors attributed the higher therapeutic efficacy of paclitaxel-containing EndoTAG™-1 to the delivery of more drug to angiogenic blood vessels. This might result in a decrease in tumor vessel density and a reduction in tumor microcirculatory perfusion index in these animals. Schmitt-Sody and colleagues (77) also emphasized that, in a dorsal skinfold model, vascular targeting of paclitaxel was achieved by encapsulating the drug in cationic liposomes. They also showed that paclitaxel-containing cationic liposomes were able to reduce *in vivo* growth and metastasis of A-Mel-3 mouse melanoma to a significantly greater extent than free paclitaxel. Eichhorn *et al.* (78) investigated the therapeutic efficiency of camptothecin encapsulated in cationic liposomes (EndoTAG™-2) and demonstrated that EndoTAG™-2 showed remarkable antitumor efficiency in

Table II Selected List of Cationic Liposomes that Have Received Evaluation *In Vivo* for Delivery of Anticancer Drugs

Encapsulated anticancer drug	Liposomal composition (molar ratio)	Tumor	Therapeutic effect
Paclitaxel	DOTAP/DOPC (25/23.5)	Humanized A-375 melanoma	Suppression of tumor angiogenesis Inhibition of tumor growth Increased survival time (75)
Paclitaxel	DOTAP/DOPC (25/23.5)	Amelanotic melanoma (A-Mel-3)	Enhanced tumor accumulation Tumor growth suppression Inhibition of local lymph node metastasis (77)
Paclitaxel	DOTAP/DOPC (100/94)	A-Mel-3	Retardation of tumor growth Decreased tumor vessel density Reduction in the microcirculatory perfusion index (76)
Paclitaxel	DOTAP/DOPC (100/94)	A-Mel-3	Increase of platelet adherence in tumor microvessels Acute impairment of the microcirculation Induction of microthromboses within the tumor microcirculation Decreased microcirculatory perfusion index (89)
Etoposide	Lecithin/CHOL/Stearylamine/ α-tocopherol (7/2/2/1)	Solid fibrosarcoma	Decreased tumor growth Prolongation of survival time Decreased cytotoxicity (74)
Cisplatin	HSPC/CHOL/TRX-20 (50/42/8)	Osteocarcinoma (Chondroitin Sulfate-expressing tumors)	Suppression of tumor growth Enhanced intra-tumoral accumulation Reduced systemic toxicity Prolongation of the survival time (88)
Doxorubicin	EPC/CHOL/DDAB (40/40/20)	Human oral carcinoma	Increased intra-tumoral accumulation Increased lifespan (79)
Doxorubicin	DOPC/DOTAB/CHOL/DOPE-PEG (50/35/10/5)	Human pancreatic cancer	Improved uptake of cationic liposomes by tumor endothelium Enhanced growth inhibitory properties (1)
Camptothecin	DOTAP	Lewis lung carcinoma (LLC)	Tumor growth suppression Decreased metastasis Reduction of tumor microvessel density Impairment of tumor microcirculation function (78)
Oxaliplatin	HSPC/CHOL/DC-6-14/mPEG$_{2000}$-DSPE (2/1/0.2/0.2)	Mouse melanoma B16Bl6	Selective binding to tumor angiogenic vessels Inhibition of tumor angiogenesis Decreased binding to erythrocytes (51)
Oxaliplatin	HSPC/CHOL/DC-6-14/mPEG$_{2000}$-DSPE (2/1/0.2/0.2)	LLC	Enhanced uptake by both tumor endothelial cells and tumor cells Tumor growth suppression Prolongation of the survival time (64)

Lewis lung carcinomas (LLC) implanted in mice. In addition, they correlated such efficient antitumor activity with a significant reduction of microvessel density. Wu *et al.* (79) demonstrated the therapeutic efficacy of doxorubicin (DXR)-containing cationic liposomes in a murine leukemia model. DXR-containing cationic liposomes showed antitumor activity superior to either DXR-containing neutral liposomes or free DXR. The authors attributed this superior antitumor activity to the higher accumulation of cationic liposomal DXR in tumors compared with that of free DXR and DXR-containing neutral liposomes.

Abu Lila *et al.* (51) recently addressed the utilization of PEG-coated cationic liposomes to selectively deliver an encapsulated anticancer drug to tumor angiogenic vessels,

and showed that PEG-coated cationic liposomes loaded with oxaliplatin (l-OHP) strongly suppressed tumor angiogenesis in a murine dorsal air sac model. Neither free l-OHP nor l-OHP encapsulated in PEG-coated neutral liposomes showed such a strong suppressive effect. In another study (64), they investigated the therapeutic efficacy of l-OHP-containing PEG-coated cationic liposomes in an LLC-bearing mice model. Treatment of the mice with l-OHP-containing PEG-coated cationic liposomes resulted in a significant suppression of tumor growth and a significant increase in survival times, relative to either free l-OHP- or l-OHP-containing neutral liposomes. Such enhanced antitumor activity was attributed to the preferential accumulation of l-OHP-containing cationic liposomes in both tumor endothelial cells and tumor cells, compared to either free l-OHP- or l-OHP-containing neutral liposomes.

Effect of Dosing Schedule on the Antitumor Efficacy of Cationic Liposomal Formulations

Despite the fact that the antitumor efficacy of anticancer drugs encapsulated in neutral or anionic liposomes has been confirmed as dependent on the dosing schedule, little is known about the impact of the dosing schedule on the anti-angiogenic efficacy of cationic liposomal formulations. Eichhorn *et al.* (80) were the first to investigate the impact of the dosing schedule on the anti-angiogenic activity of EndoTAG™-1 (paclitaxel-containing cationic liposomes). They showed that a single weekly dose was less efficient, compared to a metronomic dosing, with three to five intravenous applications per week at a lower dose. This strongly relates to the endothelial cell turnover time in solid tumors. The minimal doubling time of the tumor endothelium is approximately 2.5 days in solid mouse tumors (81). Such rapid endothelial cell turnover was assumed to compensate for the anti-vascular effect of EndoTAG™-1, administered only once a week. In fact, on this dosing schedule, tumor microvessel density was unchanged. An improved therapeutic effect with EndoTAG™-1 was achieved by drug application every 2–4 days, which is in accordance with the endothelial turnover time (~2.5 days).

Recently, Abu Lila *et al.* (71) addressed the impact of the dosing schedule on the antitumor activity of l-OHP-containing PEG-coated cationic liposomes. They emphasized that the intra-tumoral accumulation of l-OHP-containing PEG-coated cationic liposomes is dependent on the dosing schedule. Administering liposomal l-OHP every 4 days significantly enhanced the intra-tumoral accumulation of subsequently injected PEG-coated cationic liposomes, and thereby increased the therapeutic efficacy. In contrast, administration of liposomal l-OHP once a week resulted in lower antitumor activity, compared to a 4-day administration schedule. They assumed that this difference in the therapeutic efficacy between the dosing regimens (*i.e.*, 4-day *vs.* 1-week dosing schedules) may be correlated with the degree of tumor angiogenic vessel maturation. As shown earlier, cationic liposomes could selectively bind to the newly formed (immature) tumor angiogenic vessels, but not to the pre-existing mature blood vessels (1,51). One week might be enough for the maturation of tumor angiogenic vessels. Cationic liposomes might, therefore, lose their binding sites in the solid tumor. Consequently, the therapeutic efficacy of l-OHP-containing PEG-coated cationic liposomes administered once a week was lower than that administered every 4 days.

Dual Targeting of Both Tumor Endothelial Cells and Tumor Cells

Many studies have focused on the application of various combination treatment regimens that include cytotoxic and anti-angiogenic agents to improve the overall antitumor response in preclinical models (82–85). However, preclinical and clinical studies with such combinations have indicated that their toxicity profile differs from that of conventional single chemotherapy, thus ruling out additive toxicity as a major limitation of combination chemotherapy (86,87). One successful approach to improve the therapeutic outcome of either cytotoxic or anti-angiogenic agents, while minimizing the associated side effects, was to encapsulate either agent into a liposomal drug delivery system. Targeting of an anticancer agent to tumor vasculature by means of a liposomal drug delivery system has been proven to increase the therapeutic index of the agent without increasing the side effects (75,76,88,89). Moreover, the targeting of liposomal anticancer drugs directly to tumor cells by targeted liposomal delivery has also been confirmed to enhance the therapeutic efficacy and reduce side effects (90–93). Accordingly, it is easy to imagine that a strategy that targets both the tumor vasculature and the tumor cells using targeted liposomes would be more effective than a strategy that targets either tumor vasculature or tumor cells alone, which can leave a cuff of unaffected tumor cells.

Pastorino *et al.* (94) provided the proof-of-principle study for the hypothesis that the combined administration of liposomal anticancer drugs, which target tumor cells and tumor vasculature, improves therapeutic efficacy relative to each therapy used individually. To target tumor vasculature, DXR-loaded liposomes were modified with NGR peptides that target the angiogenic endothelial cell marker aminopeptidase N (2). To target tumor cells, they used anti-GD2 monoclonal antibody against the disialoganglioside receptor GD2, which is widely expressed on cancer cells of neuronal origin (95). In an orthotopic neuroblastoma xenograft model, the combined formulations showed

superior antitumor efficiency over both liposomal formulations when administered separately. They attributed such enhanced antitumor activity to the complementary modes of action of the two therapeutic approaches: DXR-loaded liposomes modified with NGR peptides acting primarily on the tumor vasculature, and DXR-loaded liposomes modified with anti-GD2 monoclonal antibody mainly affecting tumor cells. In this way, an effective 'two-compartment' tumor therapy was realized, which affected both the tumor cell and the vascular compartment within the tumor.

As described earlier, the authors recently developed a PEG-coated cationic liposome and confirmed that it is a promising carrier for the delivery of an encapsulated chemotherapeutic agent to tumor endothelium (51). Later, in another study (64), they demonstrated that in a murine solid tumor model, l-OHP-containing PEG-coated cationic liposomes showed antitumor activity superior to either free l-OHP- or l-OHP-containing PEG-coated neutral liposomes. This superior antitumor activity was confirmed to be due to the delivery of l-OHP to dual targets, tumor endothelium and tumor cells, by means of PEG-coated cationic liposomes (Fig. 1C). Such a dual targeting approach, with a single liposomal anticancer drug formulation, has the potential to overcome some of the major shortcomings of conventional strategies.

Clinical Applications of Cationic Liposomes in Cancer Therapy

The success achieved in the preclinical models provides a strong rationale for the use of cationic liposomal cytotoxic therapeutic agents for the treatment of human cancer. To date, cationic liposomal paclitaxel has been the most extensively evaluated in the clinical setting (96). The first clinical trial (97) was performed to evaluate the safety of EndoTAG™-1 in patients suffering from advanced metastatic colorectal cancer. Approximately 13% of the patients under study showed stable disease, and the treatment was well tolerated. A phase 1b clinical trial (98) was conducted to evaluate the safety and antitumor efficacy of EndoTAG™-1 in patients with metastatic breast cancer and tumor progression after anthracycline-based chemotherapy. The overall assessment of tumor response showed 6% partial response and 28% stable disease in patients receiving a dose of 0.55 mg/kg on days 1, 3, and 5 of a 3-week cycle. Nausea and vomiting were the major side effects associated with the treatment. A clinical phase II study was conducted to investigate the safety and efficacy of EndoTAG™-1 in combination with standard gemcitabine treatment in patients with locally advanced and/or metastatic pancreatic cancer (99). Two-hundred patients have been enrolled in this phase II study, and preliminary data confirm a favorable safety profile for EndoTAG™-1 in combination with gemcitabine treatment. Moreover, the study has shown promising preliminary therapeutic results, as the median overall survival was increased by EndoTAG™-1 combination therapy compared to gemcitabine standard monotherapy (100). Such data extracted from the preclinical and clinical studies could potentially serve as a basis for the future development of cationic liposomal drug delivery systems for cancer treatment. The achievements, and any limitations, of these clinical trials should encourage researchers to invest their efforts in the development of cationic liposomal formulations amenable to clinical applications.

SUMMARY AND FUTURE PERSPECTIVES

Targeting of tumor vasculature is considered a rational alternative to interstitial tumor targeting, because several factors favor this approach. First, tumor vasculature is more accessible to circulating therapeutics than tumor cells (53). Second, many cancer cells depend upon a few endothelial cells for their growth and survival, and, therefore, the death of a single endothelial cell may result in the death of more than 100 tumor cells (101,102). Third, since vascular-targeted agents need not penetrate deeply within the tumor interstitium to exert their therapeutic effect, physiological barriers to tumor targeting, such as high interstitial fluid pressure and tumor hypoxia, pose little threat to vascular targeting strategies (60,103). Fourth, endothelial cells are genetically stable; hence, multidrug resistance is not a competing factor (14). Finally, the therapeutic target, tumor vasculature, is independent of the type of solid tumor, so the killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Nonetheless, despite the many advantages of vascular targeting, a strategy that targets both the tumor vasculature and the tumor cells themselves must be more effective than strategies that target only the tumor vasculature, because this strategy can leave a cuff of unaffected tumor cells at the tumor periphery that can subsequently re-grow and kill the animal (104).

Cationic liposomes have been shown to preferentially target the tumor angiogenic microvessels of solid tumors (61–63). Therefore, cationic liposomes appear to be one of the most promising drug carriers to direct chemotherapeutic agents to the tumor endothelium to realize the vascular targeting therapy concept (75–78). This novel therapeutic strategy was first realized by the synthesis of EndoTAG™-1 (formerly known as Lipopac/MBT-0206), comprised of paclitaxel encapsulated in cationic liposomes. EndoTAG™-1 has been shown to induce endothelial cell apoptosis and severe impairment of functional tumor microvasculature (105), by triggering intravascular thrombosis within treated

tumors (89). Moreover, treatment with EndoTAG™-1 significantly retarded tumor growth and delayed the incidence of metastatic disease in subcutaneously growing experimental tumors (80). Because of these promising results, EndoTAG™-1 has entered clinical phase II for the treatment of different tumor entities. In addition to paclitaxel, to realize vascular targeting therapy in preclinical animal models, DXR, 5-FU, camptothecin, and l-OHP also have been successfully encapsulated in cationic liposomes (51,76,78,79). To date, anti-vascular tumor therapy, as monotherapy, has failed to provide convincing results in clinical trials. Anti-angiogenic drugs and vascular targeting agents cannot completely eradicate tumors, and remarkable antitumoral effects can be achieved, in the clinical situation, only by combining anti-vascular tumor therapy with conventional cytotoxic radiotherapy or chemotherapy directly targeting the tumor cell compartment. Accordingly, the recently proposed dual targeting approach—vascular targeting and tumor targeting with a single liposomal anticancer drug formulation—may have the potential to overcome some of the major limitations of conventional strategies.

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Anti-PEG IgM production by siRNA encapsulated in a PEGylated lipid nanocarrier is dependent on the sequence of the siRNA

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ABSTRACT

We recently reported that the prolonged circulation property of PEGylated cationic liposomes containing nucleic acids disappears, if the second dose is injected within a few days later, due to the production of anti-PEG IgM. This accelerated blood clearance is a concern for treating diseases which require repeated treatment with a PEGylated formulation containing nucleic acids. In this study, we investigated the effect of encapsulation of siRNA in a recently introduced PEGylated lipid nanocarrier for which the term "wrapsome" (PEGylated wrapsome, PEG-WS) was proposed as well as the sequence of the encapsulated siRNA on anti-PEG IgM production. siRNA encapsulated in PEG-WS produced little anti-PEG IgM relative to siRNA in conventional PEGylated lipoplexes. The sequence of siRNA in the PEG-WL dramatically affected the anti-PEG IgM production; a potent immune stimulatory siRNA induced a higher anti-PEG IgM production. Such enhanced effect was abrogated by incorporation of 2′-O-methyl (2′-OMe) uridine into the sequence of siRNA, probably via inhibiting cytokine induction such as IL-6 and TNF-α. Our results strongly indicate that the use of an encapsulation-type lipid nanocarrier with a low immuno-stimulatory siRNA may allow repeated dosing of siRNA containing PEGylated formulations without the induction of a strong immune reaction against PEG and thus may advance synthetic siRNA into a broad range of therapeutic applications.

1. Introduction

Small interfering RNA (siRNA) has a sequence-specific and potent gene silencing effect based on a RNA interference mechanism. In a growing number of studies the application of siRNAs as potential therapeutic agents for treating a variety of diseases, including cancers, genetic disorders and viral infections [1–4]. However, due to the instability of siRNA as well as their poor cellular uptake and pharmacokinetic profiles *in vivo*, the therapeutic application of siRNA is largely dependent on the development of a delivery vehicle which must be efficient, safe and allow repeated administration. Cationic liposomes have been studied as a promising non-viral carrier of nucleic acids including pDNA, antisense oligonucleotides and recently siRNA [5,6]. These cationic carriers strongly interact electrostatically with nucleic acids yielding a net positively charged particle. This cationic lipoplex is believed to be efficiently taken up by cells due to electrostatic interaction with the negatively charged membrane, resulting in *in vitro* transfection efficiencies. However, the cationic lipoplex makes aggregates easily with anionic serum proteins following systemic injection, resulting in accumulations in first-pass organ such as lungs and livers.

To circumvent such problem, strategies have been developed. These include the use of polyethylene glycol (PEG) to shield the positive charge on the lipoplex surface as well as the use of neutral lipids to deliver siRNA systemically. Cationic liposome-polycation-DNA (LPD) [6], stable nucleic acid lipid particles (SNALP) [7] and wrapsome (WS) [8], mainly coating of a core containing siRNA with a lipid layer including PEG-conjugated lipid, were introduced and achieve high encapsulation efficiency and long circulation of siRNA, resulting in *in vivo* therapeutic effects. These are modified by PEG-conjugated lipid (PEGylation) to achieve their prolonged *in vivo* circulation time. It is generally believed that liposome surface-grafted PEG can attract a water shell around the particle and results in reduced adsorption of opsonins and the recognition of the liposomes by the cells of the mononuclear phagocyte system [9,10]. The technology has already been utilized for anti-cancer drugs in clinical settings, exploiting the prolonged blood circulation time of PEGylated lipid nanocarriers of approximately 100 nm in diameter and their preferential accumulation in solid tumors due to the enhanced permeability and retention (EPR) effect [11]. However, we and others have reported that an intravenous injection of PEG-coated liposomes causes a second dose of PEG-coated liposomes, injected a few days later, to lose its long-circulating characteristics and to accumulate extensively in the liver. This effect is known as the "accelerated blood

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clearance (ABC) phenomenon" [12–14]. On the basis of our recent results [15,16], we proposed the following tentative mechanism for the cause of this phenomenon: anti-PEG IgM, which is produced in the spleen in response to a first dose, selectively binds to the PEG of the second dose liposomes injected several days later and subsequently activates the complement system, as a consequence, the liposomes are taken up by the Kupffer cells in the liver.

The ABC phenomenon involving anti-PEG IgM production may also represent a major concern in designing an efficient siRNA delivery system. In fact, we very recently reported that the intravenous injection of a PEG-coated siRNA/cationic liposome complex (PEGylated conventional lipoplex), prepared according to conventional methods, caused anti-PEG IgM production and thereby led to accelerated blood clearance of a second dose [17]. Many papers acknowledge that siRNA is a potent activator of the innate immune system and consequently produces inflammatory cytokines [18,19]. In addition, it is shown that the induction of innate immunity by siRNA is dependent on siRNA structure and sequence [20]. Accordingly, it is assumed that siRNA in the PEG-coated conventional lipoplex might affect anti-PEG IgM production via activation of innate immunity. Therefore, in this study, we attempted to confirm the effect of siRNA, either containing immune stimulatory sequences or not, in the lipoplex on anti-PEG IgM production. In addition, we investigated whether encapsulation of siRNA into the core of PEGylated WS (PEG-WS) attenuates anti-PEG IgM production. Our findings raise important concerns regarding the development of a potent siRNA delivery system allowing for safe and repeated administrations.

2. Materials and methods

2.1. Materials

2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000 (PEG_{2000}-DSPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were generously donated by NOF (Tokyo, Japan). A cationic lipid, *O,O'*-ditetradecanoyl-*N*-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical Co. Ltd. (Tokyo, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

2.2. Animals

Male Std-ddY mice aged 4–5 weeks (20–25 g) were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under pathogen-free conditions. All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

2.3. siRNAs

The unmodified siRNAs and the 2'-*O*-methyl uridine-modified (2'-OMe) siRNA were chemically synthesized by Nippon EGT (Toyama, Japan). The sequences of the siRNAs [17,20,25] were listed as follows: siRNA for GFP; sense sequence, 5'-GGCUACGUCCAGGAGCGCATT-3'; anti-sense sequence, 5'-UGCGCUCCUGGACGUAGCCTT-3': VEGF; sense sequence, 5'-CAUGGGACUUCUGCUCUCCTT-3'; anti-sense sequence, 5'-GGAGAGCAGAAGUCCCAUGTT-3': Argonaute2, which associates with small RNAs that guide mRNA degradation, translational repression, or a combination of both; sense sequence, 5'-GCACGGAAGUCCAUCUGAAUU-3'; anti-sense sequence, 5'-UUCAGAUGGACUUCCGUGCUU-3': firefly luciferase; sense sequence, 5'-CUUACGCUGAGUACUUCGATT-3'; anti-sense sequence, 5'-UCGAAGUACUCAGCGUAAGTT-3': inverted sequence of firefly luciferase (inverted luciferase); sense sequence, 5'-AGCUUCAUAAGGCGCAUGCTT-3'; anti-sense sequence, 5'-GCAUGCGCCUUAUGAAGCUTT-3': ApoB-1; sense sequence, 5'-GUCAUCACACUGAAUACCAAU-3'; anti-sense sequence, 5'-AUUGGUAUUCAGUGUGAUGACAC-3': β-galactosidase (β-gal); sense sequence; 5'-CUACACAAAUCAGCGAUUUUU-3'; anti-sense sequence, 5'-AAAUCGCUGAUUUGUGUAGUU-3'.

In this study, the sequences of Inverted luciferase, ApoB-1 and β-gal were 2'-OMe modified, and then, these all nucleotides except for overhung sequences were modified.

The complementary anti-sense and sense strands in TE buffer (10 μM Tris–HCl, 1 μM EDTA (pH 8.0), DNase and RNase free grade (Nippon Gene, Tokyo, Japan)) were mixed in equal amounts, followed by heating at 90 °C for 1 min. The reaction mixture was then allowed to cool at room temperature. Quality of duplex siRNA was checked by 15% PAGE. The final concentration of the duplexes was adjusted to 50 μM with TE buffer.

2.4. Preparation of cationic liposome and PEGylation on the prepared liposome

Cationic liposomes were prepared as described before [17]. Briefly, the lipids (DC-6-14/POPC = 50/50, molar ratio) were dissolved in chloroform. After evaporation of organic solvent, the resulting thin lipid film was hydrated in 9% sucrose to produce multilamellar vesicles (MLVs). The MLVs were sized by repeated extrusion through polycarbonate membrane filters (Nuclepore, CA, USA) with consecutive pore sizes of 400, 200, 100 and 80 nm. The mean diameters and zeta potentials of the resulting liposomes were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The mean diameter and zeta potential of cationic liposomes were 80.2 ± 3.2 nm and 23.4 ± 3.5 mV (n = 3). For PEGylation of cationic liposomes, a post-insertion technique was used [21]. PEG_{2000}-DSPE (5 mol% of total lipid) in 9% sucrose solution was mixed with the cationic liposomes, vortexed for 15 s and gently shaken for 1 h at 37 °C. The mean diameter and zeta potential of PEGylated cationic liposomes were 84.3 ± 2.1 nm and 18.2 ± 1.9 mV (n = 3), respectively. The concentration of phospholipids was determined by colorimetric assay [22].

2.5. Preparation of core-lipoplex

Core-lipoplex was prepared as described previously with minor modification [17]. To formulate the core-lipoplex, siRNA solution (17 μg siRNA diluted in 170 μl of 9% sucrose solution) was mixed with an equal volume of PEGylated cationic liposomes (7.14 μmol) prepared as described above and incubated for 20 min at room temperature. The mean diameter and zeta potential of core-lipoplexes were 284.1 ± 9.2 nm and 11.5 ± 4.1 mV, respectively.

2.6. Preparation of lipid-wrapped core-lipoplex (PEG-WS)

PEG-WS containing siRNA were prepared as described previously with minor modification [7,23]. To wrap the core-lipoplex with a neutral lipid layer containing PEGylated lipid, the core-lipoplex solution (340 μl) prepared as described above was mixed with 566 μl of an ethanol solution containing POPC (34 μmol) and PEG_{2000}-DSPE (1.7 μmol). The lipid ratio of the core-lipoplex to outer lipid layer was set at 1:5 (w/w) and the prepared PEG-WS contained on average 17 μg of siRNA in 42.84 μmol total lipid. The solution was diluted with 19 ml distilled water with stirring. Following centrifugation (30,000 × g, 4 °C, 1 h) and subsequent removal of supernatant containing free lipid, the pellet (siRNA-containing PEG-WS) was suspended in a small amount of 9% sucrose solution to provide an appropriate preparation. The mean diameter and zeta potential of the prepared PEG-WS were 304.6 ± 7.3 nm and −12.4 ± 1.8 mV (n = 3), respectively.

2.7. Detection of anti-PEG IgM

At day 5 after injection of siRNA-containing PEG-WS, the peripheral blood was withdrawn from each treated mouse by heart puncture. To obtain serum, the blood was placed for 30 min at room temperature and then centrifuged at 3000 rpm at 4 °C for 15 min. The serum collected from naïve mice was used as control serum. A simple ELISA as described previously [17] was employed to detect anti-PEG IgM in the serum. Briefly, 10 nmol of PEG_{2000}-DSPE in 50 μl ethanol was added to each well of a 96-well plate. The plate was allowed to air dry completely for 2 h. The lipid-coated plates were then blocked for 1 h with Tris-buffered saline (pH 7.4) containing 1% BSA and were subsequently washed three times with phosphate buffered saline (PBS, pH 7.4). Diluted serum samples (1:100) (100 μl) were then applied in the well, incubated for 1 h and washed three times with PBS. Horseradish peroxidase (HRP)-conjugated antibody (100 μl, 1 μg/ml, Goat anti-mouse IgM IgG-HRP conjugate; Bethyl Laboratories, TX, USA) was added to the wells. After 1 h incubation, the wells were washed three times with PBS. The coloration was initiated by adding 100 μl of *o*-phenylenediamine (1 mg/ml) (Sigma, MO, USA). After 15 min incubation, the reaction was stopped by adding 100 μl of 1 M H_2SO_4. The absorbance was measured at 490 nm using a microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science). All incubations were performed at room temperature.

2.8. Measurement of inflammatory cytokine

At appropriated times after injection of siRNA-containing PEG-WS (10 μg siRNA/mouse), the peripheral blood was withdrawn from each treated mouse by heart puncture. The serum was obtained as described above. Interleukin 6 (IL-6) and interferon gamma (INF-γ) and tumor necrosis factor alpha (TNF-α) in the serum was measured with Quantikine Immunoassay Kit (R&D Systems, MN, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

All values are expressed as the mean ± S.D. Statistical analysis was performed with a two-tailed unpaired Student's *t* test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p<0.05$.

3. Results

3.1. Effect of encapsulation of siRNA on anti-PEG IgM production

Anti-PEG IgM production was assessed on day 5 after a single injection, by which time the ABC phenomenon is markedly manifest [17]. We confirmed that a low-dose single injection of siRNA-containing PEGylated lipoplex, regardless of the complex type (PEGylated lipoplex (core-lipoplex)) and the encapsulation type (PEG-WS), causes a significant induction of anti-PEG IgM production (Fig. 1). Consistent with our earlier observations [17,24], the level of specific IgM induction was reversely related to the dose of both siRNA-containing lipoplexes. But, the responses against encapsulation type (WS) was completely diminished at higher dose as previously observed in the empty PEGylated cationic liposome [17,24] causing immunological tolerance, while the responses against the complex type (PEGylated lipoplex) was not declined upon increasing the dose. It appears that encapsulation of siRNA in the core of lipoplex is sufficient to attenuate activation of innate immunity by siRNA which induces the anti-PEG IgM production.

3.2. Effect of siRNA-sequence on anti-PEG IgM production

We then investigated whether the sequence of siRNA in the PEG-WS affects the anti-PEG IgM production. Two siRNAs were compared:

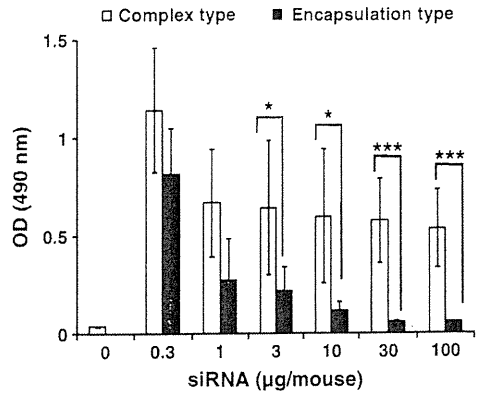

Fig. 1. Effect of encapsulation of siRNA in PEGylated lipoplex on anti-PEG IgM production. PEGylated lipoplex (core-lipoplex (complex type)) or WS (encapsulation type) containing GFP-siRNA was intravenously injected into mice. Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). * $p<0.05$ and *** $p<0.005$.

siRNA against β-galactosidase (β-gal) which has an immune stimulatory effect and produces many inflammatory cytokines [20] and siRNA against GFP which does not show a strong immune stimulatory effect in Fig. 1 and our earlier study [17].

As is shown in Fig. 2, at a low dose of 0.3 μg siRNA per mouse, GFP-siRNA-containing PEG-WS and β-gal-siRNA-containing PEG-WL produced nearly identical amounts of anti-PEG IgM. However, at doses higher than 1 μg siRNA, anti-PEG IgM production by the β-gal-siRNA-containing PEG-WL exceeded that produced by the GFP counterpart by a factor of 3 to 5. To extend the study, we assayed anti-PEG IgM levels elicited by PEG-WSs containing a range of different siRNAs. As demonstrated in Fig. 3, the potential of PEG-WS-encapsulated siRNAs to elicit anti-PEG IgM varies largely with the RNA sequence. Therefore, it is to be expected that the severity of the ABC phenomenon upon repeated administration of this type of siRNA formulation will also strongly depend on the sequence of the used siRNA. Furthermore, it is worthy here to note that an immune stimulatory siRNA such as β-gal siRNA can strongly stimulate the immune system to produce anti-PEG IgM despite the condition that the siRNA was encapsulated in the delivery vehicle.

3.3. Effect of 2′-OMe siRNA on anti-PEG IgM production

There is reason to believe that the response of innate immunity to synthesized siRNA is Toll-like receptor (TLR)-mediated [19]. Recently, some reports demonstrated that chemical modifications of the 2′-OH

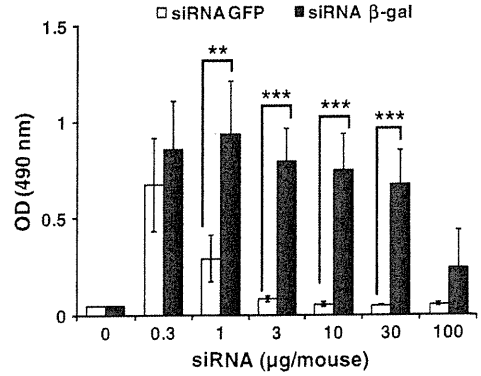

Fig. 2. Effect of immune stimulatory siRNA in PEG-WS on anti-PEG IgM production. WS containing beta-gal siRNA or GFP-siRNA was intravenously injected into mice. Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). ** $p<0.01$ and *** $p<0.005$.

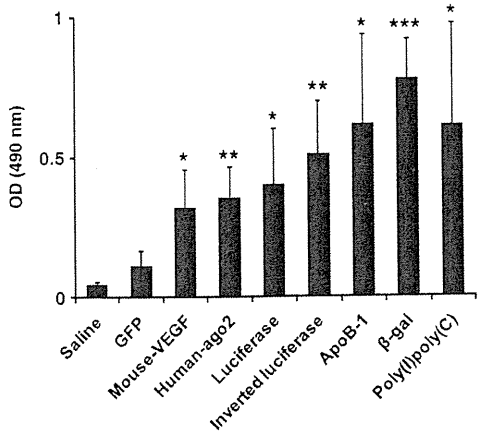

Fig. 3. Effect of sequence of siRNA in the PEG-WS on anti-PEG IgM production. WS containing various sequences of siRNA was intravenously injected into mice (10 μg siRNA/mouse). Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). * $p<0.05$, ** $p<0.01$, and *** $p<0.005$.

group in the ribose sugar backbone such as 2′-*O*-methylytion (2′-OMe) prevent recognition of the siRNA by the innate immune system without loss of RNAi activity [25,26]. Robbins et al. [27] clearly demonstrated that 2′-OMe siRNA acts as TLR7 antagonist. Therefore, we obtained 2′-OMe siRNAs against inverted luciferase, ApoB-1 and β-gal and investigated the effect of this modification of siRNA on the anti-PEG IgM production induced by siRNA-containing PEG-WS. The immune stimulatory β-gal siRNA [20] in the WS strongly induced anti-PEG IgM production, while the chemically modified β-gal siRNA, notably 2′-OMe β-gal siRNA, in the PEG-WS did not induce IgM production (Fig. 4). Similar tendency was observed with inverted luciferase siRNA and ApoB-1 siRNA. The extent of immune activation of 2′-OMe β-gal siRNA was similar to that of less immune stimulatory GFP-siRNA (Fig. 3).

To check the strength of the immune stimulatory effect of siRNA on anti-PEG IgM production, the inflammatory cytokines, IL-6, INF-γ and TNF-α, were determined. PEG-WL containing β-gal siRNA induced IL-6 at 4 h after injection, while no such induction was observed for other siRNA formulations including 2′-OMe β-gal siRNA (Fig. 5A). Similar IL-6 induction was observed for Apo B-1 siRNA, while no such induction was observed for 2′-OMe Apo B-1 siRNA (Fig. 5B). 2′-OMe

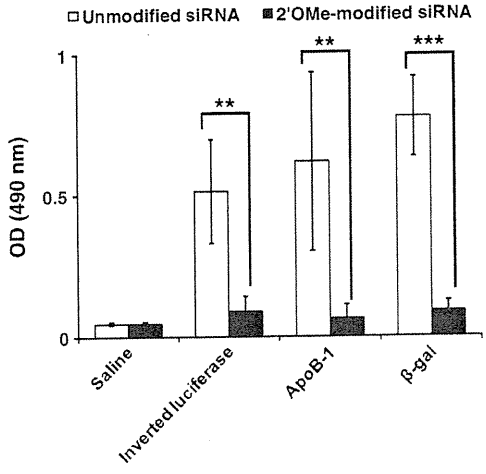

Fig. 4. Effect of 2′-*O*-methyl uridine-modification to siRNA on the anti-PEG IgM production. siRNA containing PEG-WS was intravenously injected into mice (10 μg siRNA/mouse). Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). * $p<0.05$ and *** $p<0.005$.

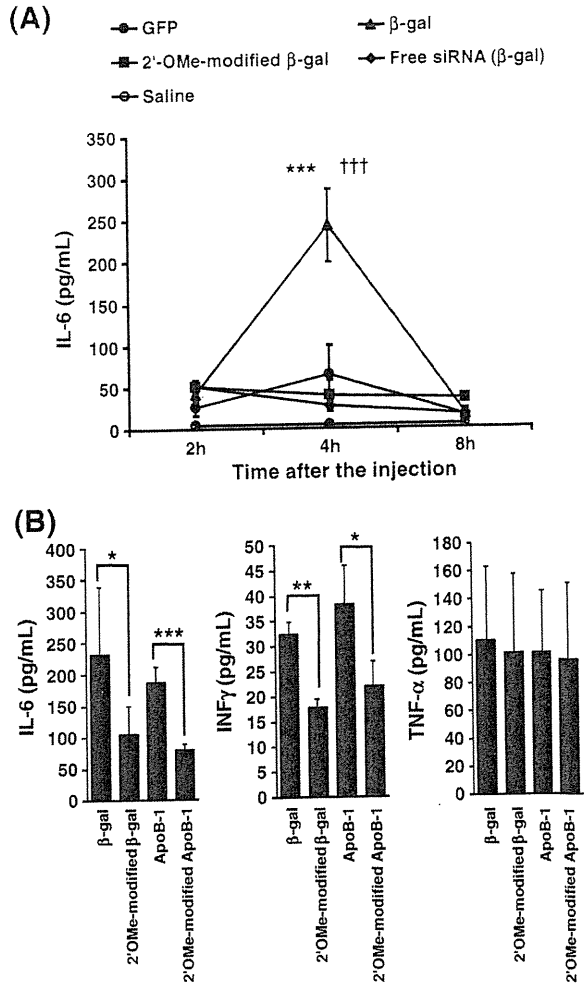

Fig. 5. Detection of inflammatory cytokine (IL-6, INF-γ, TNF-α) induced by siRNA-containing PEG-WS. (A) Time course change of IL-6 secretion. Each siRNA formulation was intravenously injected into mice (10 μg siRNA/mouse), and then, at 2, 4 or 8 h after injection, blood was withdrawn and IL-6 in the serum was determined with ELISA. Each value represents the mean ± SD (n = 4). *** $p<0.005$, β-gal siRNA versus GFP siRNA; ††† $p<0.005$, β-gal siRNA versus 2′-OMe β-gal siRNA. (B) The secretion of different types of inflammatory cytokines. At 4 h after injection of each siRNA formulation (10 μg siRNA/mouse), blood was withdrawn and inflammatory cytokines (IL-6, INF-γ, TNF-α) in the serum was determined with ELISA. Each value represents the mean ± SD (n = 4). * $p<0.05$, ** $p<0.01$, and *** $p<0.005$.

modification of siRNA reduced INF-γ production induced by PEG-WL containing β-gal siRNA and Apo B-1 siRNA. Interestingly, similar TNF-α inductions were observed in PEG-WS containing siRNA and 2′-OMe siRNA (Fig. 5B), suggesting that TNF-α may not contribute to anti-PEG IgM response against PEG-WS. These observations clearly indicate that chemical modification of siRNA to avoid potent activation of innate immunity abrogates the inductive effect of siRNA on anti-PEG IgM response against PEG-WS.

4. Discussion

A major key to successful siRNA-based therapy is the development of an efficient siRNA delivery system. For this purpose, several nanocarrier systems have been explored extensively. PEGylation is generally preferred to achieve increased stability and prolonged residence time of a nanocarrier system in the circulation. In an earlier report we showed that intravenous injection of siRNA-containing PEG-coated lipoplex gives rise to anti-PEG IgM production [24] that is

responsible for rapid clearance of a second dose of liposomes, injected a few days later [13]. This can become a major concern in designing a siRNA delivery system for *in vivo* use. In this study, we investigated the effect of siRNA encapsulation into the core of PEG-WS as well as the effect of siRNA sequence and incorporation of 2′-OMe uridine into the sequence of siRNA on the anti-PEG IgM response. Encapsulation of siRNA dramatically attenuated the production level of anti-PEG IgM (Fig. 1). The use of siRNA containing an immune stimulatory sequence (e.g. siRNA against β-gal [20]) strongly promoted anti-PEG IgM production (Fig. 2). Thus, such siRNA somewhat overpowers the inhibitory effect of lipid encapsulation of siRNA on recognition by TLRs. In addition, the incorporation of 2′-OMe uridine abrogated such adjuvant effect on the anti-PEG IgM production (Fig. 4). These results suggest that the design of a better siRNA delivery system allowing for safe and repeated administration can be achieved by combining less immune stimulatory siRNA sequences, adequate chemical modification and masking the siRNA by lipid encapsulation into the core of PEGylated liposomes.

Nucleic acids are essentially potent activators that can promote an innate immune response, inducing high levels of inflammatory cytokines such as TNF-α, IL-6 and interferons (IFNs) [18,20,28,29]. Activation of the innate immune system via synthesized siRNA is assumed to represent a significant undesirable side effect *in vivo*, due to the toxicities associated with excessive cytokine release and associated inflammatory syndromes. In addition, we here indicated that immune stimulatory siRNA in the PEG-WS functions as an adjuvant which promotes anti-PEG IgM production (Fig. 2). Thus, an understanding of how to minimize such adjuvant effect of siRNA will be helpful to researchers as they attempt to develop a safe and efficient siRNA delivery system with PEGylated lipid nanocarriers for repeated administration. As described above, in addition to choosing less immune stimulatory siRNA sequences, the lipid encapsulation of siRNA was sufficient to attenuate the adjuvant effect of siRNA on the production of anti-PEG IgM at higher dose (Fig. 1). Judge et al. [20] proposed that the recognition of siRNA in their system (encapsulation type SNALP) occurs within the endosomal pathway in a manner similar to that of other immune stimulatory nucleic acids, including ssRNAs known to activate plasmacytoid dendritic cells [30,31]. The endosome is the very cellular compartment where the siRNA-sensing pattern recognition receptors such as Toll-like receptors (TLR) exist [19]. Sioud et al. [32] also emphasized the importance of endosomal localization of siRNA for the innate immunity activation. In the acidic environment of the endosome, double strand siRNA is separated into single strand RNA. Single and double strand RNAs likely activate different TLRs, and thereby induce various immune responses. Our complex type-lipoplex (conventional lipoplex) displayed large amount of siRNA on the outer surface. By contrast, our PEG-WS (encapsulation type) entrapped siRNA within its inner core and, as a result, siRNA is fully masked. Although the exact mechanism by which the WS resulted in less engagement of immune response than the conventional lipoplex is still uncertain. The WS might prevent encapsulated siRNA from interaction with TLRs in the endosome. Lipid encapsulation (not association) of siRNA is frequently used to provide protection from intravascular nuclease degradation and passive targeting to disease sites and can enhance *in vivo* delivery of siRNA [6–8,33–35]. Our current study presents an additional advantage of a lipid encapsulation type delivery strategy for siRNA.

It is well known that nucleic acids can activate mammalian innate immunity and that the strength of the response is sequence dependent. For instance, CpG motifs in the pDNA sequence activate the TLR 9 signaling pathway in immune competent cells and induce the production of a variety of inflammatory cytokines and interferons [28]. We also recently reported that pDNA containing CpG motif strongly facilitates an anti-PEG IgM response upon intravenous injection of PEGylated pDNA-lipoplexes [24]. Heil et al. [30] showed that in siRNA a GU-rich sequence activates innate immunity via TLR7 and TLR8. Judge et al., [20] indicated that the GU contents in the siRNA motif are important for its immune stimulatory effect. By contrast, Hornung et al., [18] indicated that GU contents are not necessarily required for activation of innate immunity. Taken together, the exact immune stimulatory sequences in synthesized siRNA are unclear at present [19]. In fact, the adjuvant effect of siRNAs on the anti-PEG IgM production was random and not necessarily related to a specific siRNA sequence such as GU rich motif (Fig. 2). The siRNA sequence (β-gal 728) against β-gal [20] we employed in this study has been shown to be a potent inducer of inflammatory cytokines and this siRNA is more potent at inducing anti-PEG IgM production than other siRNAs (Fig. 3). Therefore, further work will be required to confirm the precise mechanism of siRNA-mediated immune activation before development of safe and efficient siRNA delivery system can be fully achieved.

Chemical modification of synthesized siRNA has been extensively applied to increase stabilization of siRNA against rapid nuclease degradation. These modifications include modifications of the 2′-OH group in the ribose sugar backbone, such as 2′-OMe and 2′-fluoro (2′-F) substitutions, that are readily introduced as modified nucleotides during siRNA synthesis. A number of reports have shown that siRNA containing 2′-OMe [36], 2′-F [36], 2′-deoxy [37], or locked nucleic acid [38] modifications can retain functional RNAi activity, indicating that these chemical conversions can be compatible with the RNAi machinery. Judge et al. [25] showed that incorporation of 2′-OMe uridine into one strand of the siRNA duplex completely abrogated the immune stimulatory property of siRNA without disrupting its gene silencing effect. In this study, we showed that the promoted anti-PEG IgM production by β-gal siRNA was significantly reduced by using 2′-OMe β-gal siRNA (Fig. 4), suggesting that selective modification of siRNA attenuates its adjuvant effect on anti-PEG IgM production and thus would moderate the induction of the ABC phenomenon. This brings along a novel advantage in the strategy of chemical modification of siRNA with 2′-OMe uridine.

It has been reported that inflammatory cytokines and chemokines are generally correlated with the promotion of IgM production [39,40]. Especially, IL-6 and INF-γ are closely related with IgM production in B cells [41]. We showed that 2′-OMe β-gal siRNA abrogated the IL-6 and TNF-γ secretion compared to unmodified immune stimulatory β-gal siRNA [20] (Fig. 5). Robbins et al. [27] defined 2′-OMe siRNA as an antagonist of TLR 7. This led to the assumption that the adjuvant effect of β-gal siRNA (β-gal 728) on anti-PEG IgM production is closely related with the TLR 7 signaling pathway associated with induction of IL-6 and TNF-γ secretion. In the case of pDNA, we recently reported that PEG-coated pDNA-lipoplexes also enhance anti-PEG IgM production compared with PEG-coated cationic liposomes without pDNA. In the same experiment we showed that the removal of the CpG motif from pDNA, which is the ligand of TLR9, significantly reduces anti-PEG IgM production [24]. In addition, our preliminary study indicates that PEGylated liposomes containing lipopolysaccharide (LPS), which is the ligand of TLR 4, enhances anti-PEG IgM production in the same manner as siRNA and pDNA (personal communication of T.T.). These results indicate that the adjuvant effect of nucleic acids on anti-PEG IgM production is mediated via TLRs-related signaling pathways. However, since TLRs are present in various immune competent cells such as macrophages, dendritic cells and B cells, the entire event relating to the adjuvant effect of immune stimulatory nucleic acids can be expected to be very complex. Further extensive research will be required to open this black box.

5. Conclusion

We showed that the encapsulation of siRNA in PEGylated lipoplexes (PEG-WS) and the use of less immune stimulatory siRNA as a payload can achieve the abrogation of anti-PEG IgM response against the delivery vehicle, presumably due to preventing siRNA-mediated