Proteoglycans Mediate Cationic Liposome-DNA Complex-based Gene Delivery in Vitro and in Vivo*

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The factors controlling cationic liposome-DNA complex (CLDC)-based gene transfer in cells and in animals are poorly understood. We found that cell surface heparin/heparan sulfate-bearing proteoglycans mediate CLDC-based gene transfer and expression both in cultured cells and following intravenous gene delivery into animals. CLDC did not transfected Raji cells, which lack proteoglycans, but did efficiently transfect Raji cells stably transfected with the proteoglycan, syndecan-1. Fucoidan, heparin, or dextran sulfate, all of which are highly anionic polysaccharides, each blocked CLDC-mediated transfection both in cultured cells and following intravenous injection into mice, but had no effect on transfection by either recombinant adenovirus infection or electroporation. Intravenous pretreatment of mice with heparinases, which specifically cleave heparan sulfate molecules from cell surface proteoglycans, blocked intravenous, CLDC-mediated transfection in mice, confirming that proteoglycans mediate CLDC gene delivery in vivo. Modulation of proteoglycan expression may prove useful in controlling the efficiency of, as well as targeting the sites of, CLDC-based gene transfer in animals.

Cationic liposome complexation to DNA is a powerful method for gene delivery both in vitro and in vivo. Formation of cationic liposome-DNA complexes (CLDC) protects the DNA and provides a vehicle that delivers the DNA into cells (1). Advantages of CLDC-mediated transfection in vivo include low immunogenicity (2) and toxicity (3–5) compared with viral vectors (6, 7) and the potential to transfect many tissue and cell types (4, 8). However, studies of in vivo cationic liposome-mediated gene delivery by intravenous injection have shown that some tissues are better transfected than others, e.g. lung and heart (9). Furthermore, endothelial cells and resident macrophages seem to be transfected preferentially over other cell types (9). These indications of specificity pose both possibilities and problems in expressing biologically relevant genes in appropriate cells in vivo.

Understanding the mechanism of CLDC delivery into cells is important for controlling both the efficiency and the sites of gene transfer and expression. Electron microscopy studies have described some of the events of gene transfer by lipopolylysine or cationic liposome complexes (10–14), but leave the molecular mechanisms undefined. A major step toward elucidating the mechanism of CLDC delivery in cells is to identify the proteins and molecules on the cell surface responsible for uptake of CLDC. Knowledge of the functional molecules mediating CLDC uptake may result in strategies that allow efficient transfection of more tissue or cell types in vivo. Alternatively, understanding the mechanism of CLDC uptake may lead to more efficient protocols for transfecting greater numbers of cells within a given tissue. Finally, definition of the cell surface markers that bind to CLDC is essential before methods for targeting genes to specific tissues and cell types may be designed. In this paper, we focus on identifying molecules on the cell surface involved in the delivery of CLDC bearing a net positive charge across the plasma membrane both in transfected cells and following systemic delivery in animals.

Proteoglycans perform a wide variety of functions (for reviews, see Refs. 15–18) ranging from formation of extracellular matrix (19, 20) to cell-cell contact and communication (21). Proteoglycans also function in the binding and entry of many viruses into cells (see Ref. 22 for review), including herpes simplex virus (23), murine cytomegalovirus (24), and human immunodeficiency virus type-1 (25). Proteoglycans can also act as reservoirs for growth factors (26–28) and in some cases can regulate growth factor function, e.g. basic fibroblast growth factor (29–31). Illustrating the diversity of functions encompassed by these proteins, proteoglycans are also involved in the regulation of lipid metabolism (32, 33) and in the binding of monocyes to subendothelial matrix (34).

Proteoglycans have been shown to mediate gene transfer into cultured cells by methods relying on polysine or cationic liposomes (35). This observation suggests that cell surface proteoglycans may play a role in the uptake of CLDC in vivo. However, it has also been shown that soluble heparin sulfate can release DNA from CLDC in vitro, suggesting that glycosaminoglycan-bearing proteoglycans present in serum or interstitial fluids may block CLDC-based transfection by releasing DNA from CLDC and preventing internalization of the DNA (36, 37). The role of proteoglycans in mediating CLDC-based gene delivery in vivo is, therefore, unknown.

We present evidence that proteoglycans play a significant role in CLDC-mediated delivery and expression of heterologous genes both in vitro and in vivo. The proteoglycan syndecan-1 is,

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‡ The abbreviations used are: CLDC, cationic liposome-DNA complex; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; CHO, Chinese hamster ovary; FBS, fetal bovine serum; DOTAP, 1,2-dioleoyl-3-(trimethylammonio)propane; DOPE, dioleoylphosphatidylethanolamine; DOTIM, 1-[2-(9-Z)-octadecenoyloxy]-ethyl]-2-(8-Z)-heptadecenyloxy]-3-(2-hydroxyethyl)-indazolium chloride.
in particular, implicated as a mediator of gene transfer in vitro. Heparinase I pretreatment in vivo demonstrates the specific importance of heparan sulfate and heparan sulfate proteoglycans in CLDC-based, intravenous transfection in mice. The mechanism of inhibition of gene transfer and expression by pretreatment with either polysaccharides (fucoidan or heparin) or heparinase in vivo is by limiting the uptake of CLDC by cells, suggesting that proteoglycans on the cell surface play a crucial role in uptake of CLDC in vivo.

EXPERIMENTAL PROCEDURES

**Plasmids**—The construction of p2411 has been described (9). Plasmids were purified as described previously (9).

**Preparation of Cationic Liposomes and CLDC**—DOTIM:DOPE SUV and DOTIM:cholesterol MLV were prepared as described previously (9). CLDC were integrated as described (2).

**In Vivo Transfections**—For CLDC transfections in vitro, 1–2 × 10⁵ cells were plated per well in 12- or 24-well plates. Cell types used were hamster CHO cells, mouse B16 melanoma cells, the human prostate cancer lines PPC-1 and DU-145, the human breast cancer line MDA-435, Raji cells, and Raji cells stably transfected with syndecan-1. CHO cells were grown in Ham’s F-12 with 10% fetal bovine serum (FBS). B16 and PPC-1 cells were grown in RPMI-1640 with 5% and 10% FBS, respectively. DU-145 and MDA-435 were grown in Eagle’s minimal essential medium with Earle’s balanced salt solution plus 10% FBS and Liebovitz’s L15 plus 10% FBS, respectively. Raji wild type and syndecan-1 stably transfected Raji (SI-Raji) cells were a generous gift of A. Rapraeger (University of Wisconsin, Madison, WI) and were cultured in RPMI 1640 plus 10% FBS, supplemented with 300 μg/ml hygromycin B for 5 days. SI-Raji and MDA-435 cells were grown at 37°C with 5% CO₂, with the exception of MDA-435, which was grown without CO₂. Cells were transfected as described previously (9). Prior to CLDC transfection, cells were treated with fucoidan (50,000 Mₚ), dextran (40,000 Mₚ), or dextran sulfate (500,000 Mₚ) purchased from Sigma. Heparinase I and III (Sigma) were dissolved in 0.15M sodium chloride. As an additional control, heparinase I was injected at appropriate times with either phosphate-buffered saline or left untreated, and then subjected to DNase I digestion with 10–20 units of DNase I (Boehringer Mannheim) for periods of 5, 30, or 90 min. Two micrograms of DNA in 100 μl of CLDC intravenously by tail vein injection. Pretreatments of mice were also by intravenous tail vein injection. Pretreatment with either polysaccharides (fucoidan or heparin) or heparinase I pretreatment in mice showed no apparent toxicity. Pretreatment with either polysaccharides (fucoidan or heparin) or heparinase I pretreatment in mice showed no apparent toxicity.

**Electron Microscopy**—Pellets of B16 melanoma nuclei were generated by centrifugation at 500 × g for 5 min in 15-ml plastic conical centrifuge tubes. The supernatant was carefully aspirated from the pellet, which was fixed by immersion in 2% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.4, for 2 h at room temperature. The fixed pellet was rinsed with phosphate buffer, and postfixed in 2% osmium tetroxide in phosphate buffer for 1 h at room temperature. After postfixation, the pellet was rinsed in phosphate buffer, and dehydrated in a graded series of ethanol solutions (35% v/v, 50% v/v, 70% v/v, 80% v/v for 5 min each; 95% v/v, and three changes of 100% v/v for 10 min each.) After dehydration, the pellet was infiltrated with unaccelerated Durcupan ACM epoxy resin (components A/M, B, D; Fluka Chemie AG, Buchs, Switzerland) for 2 h at 60°C, then exchanged with accelerated Durcupan ACM (components A/M, B, C, D), and polymerized for 36 h at 70°C. Samples were sectioned on a Reichert-Jung ultra-microtome. Sections (80 nm) were collected and placed on 300-mesh, thin bar copper grids (EMS, Fort Washington, PA), which were then post-stained with uranyl acetate and lead citrate (10 and 15 min, respectively) at 60°C. Sections were viewed, and micrographs taken, on a Philips CM120 STEM electron microscope at 60 kV.

**Dnase Protection**—One ml of CLDC was made at a DNA to lipid ratio of 1:8, such that the final concentration of DNA was 2 μg/100 μl. CLDC were made with either DOTIM:DOPE SUV or DOTAP:DOPE SUV. One hundred microliters of the resulting complex was treated with 1 μg fucoidan or 1 μg dextran sulfate for 10 min or left untreated, and then subjected to DNase I digestion with 10–20 units of DNase I (Boehringer Mannheim) for periods of 5, 30, or 90 min. Two micrograms of DNA in 100 μl, uncomplexed to liposomes, were treated with 10–20 units of DNase I for 5 and 30 min alone or in the presence of 1 μM fucoidan or dextran sulfate. CLDC and naked DNA were made in 5% dextrose with 50 mm Tris, pH 7.4, and 0.9 mm manganese chloride to mimic the reaction conditions of DNase I treatment. No evidence of either precipitation or microaggregation of liposome-DNA complexes was observed under these conditions. One hundred microliters of each sample were extracted once with phenol:chloroform (1:1) and extracted twice with chloroform, and 50 μl were loaded on 1% TAE gels overnight. Blotting onto Hybond membrane (Amersham Pharmacia Biotech), prehybridization, hybridization, and washes were performed as described (45). A 1.2-kilobase pair HindIII/EcoRV gel-purified fragment of luciferase from p2411 was radioactively labeled using the standard Primed DNA labeling kit (Boehringer Mannheim). Nuclei were isolated from 2 × 10⁵ B16 cells as described (46), and lysed in Nonidet P-40 lysis buffer (10 mm Tris, pH 7.4, 10 mm sodium chloride, 3 mm magnesium chloride, and 0.5% Nonidet P-40). Nuclear DNA was isolated from washed nuclei using the same technique as for total DNA isolation (45).

RESULTS

**Proteoglycans Can Mediate Transfection by CLDC in Vitro**—Raji cells stably expressing syndecan-1 were transfected by CLDC in a manner dependent on the net positive charge of the complex (Fig. 1), indicating that the syndecan-1 proteoglycan functions in the uptake of CLDC in vitro. Wild type Raji cells were essentially untransfectable by CLDC (Fig. 1). However, wild type Raji cells that had been stably transfected with the syndecan-1 gene were more efficiently transfected by CLDC (Fig. 1). Furthermore, CLDC-mediated transfection of syndecan-1-bearing cells increased significantly as the complex became more positive in net charge (Fig. 1). Cells were transfected with CLDC made at ratios of 1:1, 1:2, 1:4, and 1:6 (μg of

![Fig. 1. Syndecan-1 expression renders Raji cells transfectable by CLDC. Wild type Raji cells and syndecan-1 stably transfected Raji cells were transfected with 2 μg of p2421/sample at complex ratios of 1:1, 1:2, 1:4, and 1:6. The relative luciferase activity is shown as the mean ± standard deviation of 3 replicate samples for each CLDC. Potential signal differences between the various groups was determined using a two-tailed Student’s t test.](image-url)
DNA/nmol of total lipid), and the greatest difference in CLDC-transfection efficiency between wild type and syndecan-1 stable transfectants was seen with CLDC made at a ratio of 1:6. These data showed that, in Raji cell culture, proteoglycan expression is critical for efficient CLDC-mediated transfection.

Fucoidan Inhibits in Vitro CLDC-mediated Transfection—Because the positive cationic head group of CLDC interacting with a negatively charged cell surface molecule appeared to be the mechanism of CLDC uptake, pretreatment with a polyanionic compound could interfere with transfection. Pretreatment of cells with fucoidan, heparin, or dextran sulfate, all polysulfated polysaccharides, largely blocked CLDC transfection in vitro (Fig. 2A). Both heparin and heparan sulfate have previously been reported to block transfection mediated by polylysine-DNA complexes (35). Concentrations of fucoidan, heparin, or dextran sulfate as low as 100 nM were sufficient to inhibit transfection of murine B16 cells (Fig. 2A). Dextran sulfate, which blocked transfection at 10 nM, was the most efficient inhibitor of CLDC transfection. The charge of the polysaccharide appeared to be the significant factor in inhibiting CLDC transfection. Uncharged dextran, with a molecular weight similar to that of the fucoidan, failed to inhibit transfection, and even caused a significant elevation of transfection at high concentrations (Fig. 2A). Similar levels of inhibition of CLDC-mediated transfection by fucoidan was observed in PPC-1, MDA-435, DU-145, and CHO cells as well (data not shown). A concentration of fucoidan (10 nM) that has little effect on CLDC-mediated transfection was, nevertheless, more effective at inhibiting transfection when pretreatment with fucoidan was performed on CLDC rather than on cells (Fig. 2B). Furthermore, fucoidan inhibited the transfection of cells primarily when using methods relying on positive charge, i.e. CLDC and calcium phosphate. The 1 μM level of fucoidan, which inhibited CLDC and calcium-mediated transfection, did not interfere with adenoviral or electroporation methods of transfection in vitro (Fig. 2C).

Fucoidan inhibited CLDC-mediated transfection in vitro by blocking DNA uptake into cells. Nuclei were isolated from cells untreated or pretreated with fucoidan and subsequently transfected with CLDC, and nuclear DNA was assayed for the presence of the luciferase plasmid used in making the complex. The DNA/nmol of total lipid), and the greatest difference in CLDC-transfection efficiency between wild type and syndecan-1 stable transfectants was seen with CLDC made at a ratio of 1:6. These data showed that, in Raji cell culture, proteoglycan expression is critical for efficient CLDC-mediated transfection.

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Fucoidan inhibited CLDC-mediated transfection in vitro by blocking DNA uptake into cells. Nuclei were isolated from cells untreated or pretreated with fucoidan and subsequently transfected with CLDC, and nuclear DNA was assayed for the presence of the luciferase plasmid used in making the complex.
DNA did traverse the cell membranes of untreated cells (Fig. 3). In comparison, cells pretreated for 30 min with 1 μM fucoidan did not express luciferase (see Fig. 2A) and had no luciferase DNA in their nuclei (Fig. 3). Southern analysis of total DNA from whole cell extracts failed to show evidence of any DNA crossing the cell membranes of cultured cells pretreated with fucoidan (Fig. 3). Electron microscopy showed that nuclei preparations were free of plasma membrane, indicating that extranuclear DNA bound to membranes was not a source of contaminating DNA (Fig. 3B).

DOPE or cholesterol containing cationic liposomes were capable of protecting DNA from digestion, even in the presence of fucoidan, making it unlikely that disruption of the CLDC is the mechanism by which fucoidan inhibits both DNA uptake and subsequent reporter gene expression in cells. Previously, dextran sulfate has been shown to disrupt DNA complexed to liposomes composed purely of cationic lipid (36, 37). In order to determine whether DNA was also released from CLDC prepared from a 1:1 cationic lipid:DOPE mixture, CLDC were incubated for 10 min with 1 μM fucoidan and subjected to nuclease digestion for 5, 30, and 90 min. Virtually no degradation of DNA was observed in CLDC pretreated with fucoidan at any time point (Fig. 4). Similar liposome-mediated protection of DNA was observed when CLDC were preincubated with dextran sulfate (Fig. 4). Conversely, DNA unprotected by complexing to liposomes was readily degraded by a 90-min exposure to DNase I (Fig. 4). Digestion of DNA by DNase I was complete by 5 min in the presence of fucoidan or dextran sulfate (Fig. 4), indicating that these polysaccharides did not inhibit DNase I activity under the conditions tested. Multilamellar vesicles of DOTIM:cholesterol were equally capable of protecting DNA in complexes from DNase I digestion (data not shown). However, CLDC made with pure cationic lipid (DOTAP alone) did not protect DNA from DNase I digestion whether with or without fucoidan pretreatment (data not shown), as previously reported (36, 37).

Fucoidan Inhibits CLDC-mediated Transfection following Intravenous Injection into Mice—Although it is not always true that in vitro observations are repeated in vivo (38, 39), fucoidan also inhibited luciferase expression levels in mice injected with CLDC. Mice were pretreated with fucoidan for various time periods before intravenous injection of CLDC. Twenty-four hours after CLDC injection, tissues were assayed for luciferase activity and compared with the level of luciferase expression in tissues from mice not pretreated with fucoidan. Lung and heart tissues, the tissues most efficiently transfected by intravenous injection of CLDC containing DOTIM:cholesterol MLV (9), showed drastic reductions in luciferase activity after mice were pretreated for 1 h with fucoidan (Fig. 5A). Longer pretreatments with fucoidan resulted in less significant effects on CLDC-mediated transfection efficiency, presumably due to ongoing clearance of the highly negatively charged fucoidan from the circulation. The magnitude of luciferase depression in tissues pretreated for 1 h with fucoidan in vivo is similar to that seen in vitro, i.e. approximately 100-fold. Southern analysis of total DNA from mouse lungs and livers showed decreased levels of p4241 plasmid in the tissues of animals pretreated with fucoidan for 1 h (Table I). Nuclear DNA isolated from lungs also showed a 3-fold decrease in p4241 plasmid present in fucoidan treated animals compared with untreated animals (Table I). A 1-h pretreatment with fucoidan also resulted in significantly decreased levels of rhodamine-lipid found in the lung and liver (data not shown), as assayed by extraction of total lipids from tissues and fluorometric quantitation of the rhodamine-phosphatidylethanolamine MLV. These in vivo data concur with our in vitro observations in which fucoidan pretreatment resulted in lowered levels of DNA delivered into cells by CLDC, leading to significantly reduced luciferase expression.

Proteoglycans Are Involved in CLDC Transfection in Vivo—Pretreatment of mice with heparinase I prior to intravenous CLDC injection resulted in lowered levels of luciferase expression, indicating that proteoglycans are important for intravenous CLDC transfection. Heparinase I specifically cleaves the heparan sulfate glycosaminoglycan chains on cell surface proteoglycans, and intravenous injection of heparinase I has been shown to reduce significantly proteoglycan levels in mice (33). Mice were pretreated with heparinase I by intravenous injection of a saline solution of the enzyme 15 min before CLDC injection. Control mice were pretreated for 15 min with either saline solution alone or with boiled heparinase I. The transfection efficiency of CLDC was significantly decreased in the lungs, hearts, and spleens of heparinase I-treated mice when compared with the transfection efficiency in tissues from untreated mice (Fig. 5B). Boiling and denaturing the heparinase I negated the effect of active enzyme on CLDC transfection efficiency in mice (Fig. 5B), indicating that heparan sulfate cleavage function was necessary to inhibit CLDC-mediated transfection. Pretreatment of mice with a mixture of heparinase I and heparinase III showed the same inhibition of luciferase expression by CLDC transfection in comparison to mice.
pretreated only with heparinase I (data not shown). Similar to the effect of fucoidan, heparinase I pretreatment of mice also significantly decreased the levels of rhodamine-labeled lipid recovered from lungs in pretreated mice compared with untreated mice (data not shown). Southern analysis showed 2-fold less reporter plasmid DNA in lungs from mice pretreated with heparinase I when compared with DNA levels found in control lungs (Table I). These data indicate that mice pretreated with heparinase I were compromised for taking up DNA delivered by cationic liposomes, and intact heparin and heparan sulfate glycosaminoglycans on the cell surface play a significant role in CLDC-mediated intravenous transfection in vivo.

**DISCUSSION**

Factors that appear to function by a common pathway in mediating CLDC-based gene delivery both in vitro and in vivo are especially important to identify in order to understand, control, and improve CLDC-based gene delivery. Recent data in our laboratory highlight the inability to predict consistently and accurately from in vitro results the factors involved in controlling in vivo CLDC-mediated gene transfer. Specifically, our recent data show that depletion of sialic acids, the other predominant anionic cell surface molecule, blocks in vivo transfection by CLDC, just as depletion of proteoglycans does. Conversely, depletion of proteoglycans in vitro blocks CLDC-based transfection, whereas depletion of sialic acids enhances it. This lack of correlation between in vitro and in vivo effects is unexpected and demonstrates the importance of confirming in vitro results in in vivo systems. In this paper, we have identified proteoglycans as an important mediator of both in vitro and in vivo CLDC-mediated transfection.

The role of proteoglycans in mediating the delivery of DNA by cationic liposomes in vivo, and whether that role is inhibitory or supportive, has been a subject of controversy. Experiments using both polylysine-DNA and cationic liposome-DNA complexes indicate that proteoglycans assist in the delivery of genes in vitro (35). Transfections of CHO mutant cells deficient in the display of proteoglycans on the cell surface and cells

![FIG. 5. Intravenous, CLDC-mediated transfection in vivo is inhibited by either fucoidan or heparinase I. A, mice were pre-injected with fucoidan at 1, 2, 4, and 10 h prior to CLDC injection. Luciferase activity in heart, liver, spleen and lung are given in pg luciferase/mg tissue. “CLDC only” mice were not treated with fucoidan. Mice were treated in groups of four, and values represent the mean ± standard deviation. B, mice were pre-injected with heparinase I, saline, or boiled heparinase I 15 min prior to injection of CLDC. Luciferase activities (pg of luciferase/mg of tissue) in lung, heart, and spleen are shown. Mice were treated in groups of three, and values represent the mean ± standard deviation. Each mouse experiment was repeated three times, yielding comparable results (data not shown).](image-url)

**Table I**

| Sample       | Total DNA* | p value* | Nuclear DNA* | p value* |
|--------------|------------|----------|--------------|----------|
| Lungs        |            |          |              |          |
| CLDC only    | 2,441,875  |          | 897,394      |          |
| 1 h fucoidan | 534,435    | ≤0.025*  | 293,069      | ≤0.005*  |
| 2 h fucoidan | 1,619,559  | <0.375   | 402,747      | ≤0.01*   |
| 10 h fucoidan| 1,753,617  | ≤0.375   | 1,040,976    | ≤0.375   |
| Saline/CLDC  | 544,635    | ≤0.375   | ND           |          |
| Boiled heparinase | 659,029 | <0.01*  |          |          |
| Heparinase   | 338,342    | ≤0.375   | ND           |          |
| Livers       |            |          |              |          |
| CLDC only    | 633,324    |          |              |          |
| 1 h fucoidan | 618,629    | >0.4     |              |          |
| 2 h fucoidan | 438,449    | ≤0.375   | ND           |          |
| 10 h fucoidan| 656,217    | >0.4     |              |          |
| Saline/CLDC  | 583,882    |          |              |          |
| Boiled heparinase | 330,028 | ≤0.1    | ND           |          |
| Heparinase   | 450,161    | ≤0.375   |              |          |

*Quantitation of DNA isolated from tissues was performed by phosphorimaging of a Southern blot hybridized by a luciferase probe. The figures shown are integrated volumes of luciferase gene signal and are the averages of three replicates.

* Compared to levels of DNA found in tissues from untreated or control animals. *, p values which show statistically significant reductions in test compared to control samples.

† DNA from tissues of mice treated with fucoidan prior to CLDC injection.

‡ DNA isolated from tissues of mice treated with saline, boiled heparinase I, or heparinase I for 15 minutes prior to injection of CLDC.

ND, not determined.
treated with sodium chlorate to replace the sulfate moieties on the cell surface were less efficient, indicating that proteoglycans and the sulfates on glycosaminoglycans function in the delivery and expression of DNA in vitro (35). In contrast, data have also been presented showing that polyanionic polysaccharides, including dextran sulfate and heparin, are capable of disrupting CLDC made with 100% cationic lipid and no neutral lipid in vitro (36, 37). It was hypothesized that charge interactions disrupt the structure of CLDC and result in the release of DNA from the complex. Based on these findings, Szoka and colleagues (36, 37) predicted that proteoglycans on the cell surface might hinder the uptake of DNA in vivo, because proteoglycans display polysulfated glycosaminoglycan chains, similar to the polyanionic polysaccharides heparin and dextran sulfate.

In this paper, we present evidence that polysulfated polysaccharides do not disrupt biologically active complexes used for transfection, which are made of equal molar amounts of cationic and neutral lipid (Fig. 4), in contrast to previous results obtained using liposomes composed of 100% cationic lipid to make CLDC (36). In addition, we investigated whether proteoglycans inhibited CLDC-mediated gene delivery after intravenous injection of CLDC in mice. By pretreating mice with heparinase I (Fig. 5B), an enzyme specific for the cleavage of heparan sulfate proteoglycans, we showed that intact proteoglycans are necessary for the efficient delivery of DNA to cells in the tissues of mice injected intravenously with CLDC. Further supporting the involvement of proteoglycans, we have presented direct evidence that Raji cells, which are poorly transfected by CLDC, require expression of syndecan-1 to make them transfectable by CLDC-mediated gene transfer (Fig. 1).

Consistent with the role of proteoglycans in CLDC-mediated transfection, a 1-h in vivo fucoidan pretreatment inhibits the expression of luciferase in mice intravenously injected with CLDC (Fig. 5A). Pretreatments with fucoidan for longer time points in vivo were not inhibitory to CLDC-mediated gene transfer, most likely due to the fact that this highly anionic molecule is quickly cleared from the circulation and thus has a short duration of activity. In support of the hypothesized quick clearance of fucoidan, we were unable to detect biologically active FITC-fucoidan in the circulation as early as 1 h after injection as measured by fluorescence (data not shown). We have also found that heparin abolishes CLDC-mediated gene transfer following intravenous injection of CLDC into mice as effectively as fucoidan (data not shown). Both fucoidan and heparin could bind to positively-charged CLDC, inhibiting binding to negatively charged cell surface proteoglycans. In agreement with this hypothesis, we have found that pre-incubating CLDC with fucoidan blocks transfection in vitro more efficiently than pre-incubating cells with fucoidan (Fig. 2B). Alternatively, the large polyanionic polysaccharides fucoidan and heparin, resembling extracellular matrix components, could cross-link proteoglycans of cells, making those proteins unavailable for binding to CLDC.

The inhibitory effect of the specific enzymes, heparinase I and III, on gene expression in tissues of mice following intravenous injection of CLDC indicates the involvement of proteoglycans, or at least the glycosaminoglycan chains of these cell surface proteins, in the uptake of CLDC into cells. Cleavage and release of glycosaminoglycan chains yield at least two possible mechanisms for the inhibition of CLDC uptake into cells by heparinase pretreatment. First, cells stripped of glycosaminoglycan chains by pretreatment with heparinase would be devoid of negatively charged “CLDC receptors” and consequently would be unable to bind CLDC. Alternatively, the glycosaminoglycan chains released by enzymatic cleavage in the tissues of animals pretreated with heparinase could possibly bind to CLDC and prevent the complex from contacting the appropriate “receptor” on the cell surface. In either scenario, CLDC must bind to polyanionic glycosaminoglycan chains, suggesting that the glycosaminoglycan portion of the proteoglycan is a mediator for CLDC transfection in vivo. It is interesting to note that our observed inhibition of CLDC uptake in tissues by sulfated polysaccharides is similar to the inhibition of retroviral infection of malignant pleural effusions by chondroitin sulfates (40), raising the question of whether chondroitinases may also inhibit CLDC uptake in vivo.

The precise role of proteoglycans in mediating CLDC uptake into cells both in vitro and in vivo remains to be elucidated. Proteoglycans could bind CLDC and then be internalized as a proteoglycan-CLDC complex into cells. Alternatively, proteoglycans could capture CLDC from the circulation and present the complex to a second cell surface protein or receptor, which in turn undergoes endocytosis, similar to the involvement of proteoglycans in mediating the internalization of lipase (41). There is at least one example of a proteoglycan requiring other proteins to undergo endocytosis: two receptors of 51 and 26 kDa mediate the binding and endocytosis of decorin, a plasma proteoglycan (42). In view of potentially unidentified proteins involved in CLDC uptake, it is interesting to note that fucoidan is an inhibitor of the scavenger receptor (43), suggesting the possibility that the scavenger receptor might play a role in CLDC uptake.

One can conclude from this and other studies that the proteoglycan superfamily serves as an important cell surface component for many gene delivery vectors that produce cationic DNA complexes. This being so, the interaction of the complex with the cell is primarily electrostatic, and does not involve a receptor specificity of the binding site for the cationic moiety. Therefore, the substantial differences in transfection efficiency between various cationic systems are most likely caused by differences in physical properties of the complex such as size, stability, net surface charge, or charge density. This inference is important because it points to one of the most fruitful areas for future studies of these systems, when the effects of variations in the physical properties of CLDC are assessed with respect to biological end points.

Identification of proteoglycans as an important step in the pathway of delivering CLDC into cells for expression of heterologous genes may allow the alteration of proteoglycan expression or function in vivo to control the process of in vivo CLDC-mediated gene transfer. Further investigation of the specific mechanisms of proteoglycan function and identification of other proteins involved in CLDC uptake may yield new methods for increasing the transfection efficiency or targeting the tissue and cell types transfected by CLDC-mediated gene transfer.

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