Intracellular Accumulation of Secreted Proteoglycans Inhibits Cationic Lipid-mediated Gene Transfer

CO-TRANSFER OF GLYCOSAMINOGLYCANS TO THE NUCLEUS*

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Molecules secreted by potential target cells may interfere with cationic lipid-mediated gene transfer. This has been studied using human lung fibroblasts and human epitheloid lung cancer cells. Secreted cell medium components caused a substantial decrease both in the uptake of cationic lipid-DNA complexes (2–4-fold) and in reporter gene expression (100–1000-fold). Metabolic labeling of the cell medium showed that especially [35S]sulfate-labeled macromolecules competed with DNA for binding to the cationic lipid. Release of DNA from the cationic lipid by cell medium components was demonstrated by an ethidium bromide intercalation assay. In the presence of the cationic lipid, the secreted macromolecules were internalized by the cells. By enzymatic digestions, it was shown that the competing macromolecules consist of chondroitin/dermatan sulfate and heparan sulfate proteoglycans and that the effects on transfection were mediated by the polyanionic glycosaminoglycan portion of the proteoglycan. Accordingly, pretreatment of cell medium with the polycationic peptide pantamine sulfate abrogated the inhibitory effects on gene transfer. Fluorescence microscopy studies revealed that heparan sulfate, internalized as a complex with cationic lipids, accumulated in the cell nuclei. These results support the view that the lack of specificity of this type of gene transfer vehicle is a major hindrance to efficient and safe in vivo administration.

More than 300 protocols have been approved for clinical gene therapy studies, in which roughly 20% use nonviral delivery systems (1). The use of viral vectors has potentially many risks, including toxicity associated with expression of viral structural proteins, insertional mutagenesis, and induction of different immune responses (2–4). With the advantages of being nonimmunogenic and easy to reproduce (purify to homogeneity), synthetic plasmid-liposome complexes constitute the most important alternative to viral vector systems. In particular, substantial efforts have been focused on the development of cationic lipids (CLs),† which are efficient carriers for DNA (5–6), RNA (7), and oligonucleotides (8) in vitro. A number of in vivo studies have also been performed using this type of gene delivery system (9–11).

Binding of the CL-DNA complex to the cell membrane and entry into the cell are the initial steps of the transfection process. The ratio of CL to DNA is known to be important for optimal efficiency in vitro (5). The optimal ratio occurs when the number of positive charges incorporated into the CL exceeds the number of negative charges on the DNA, which reflects a necessary electrostatic association of the CL-DNA aggregates with the negatively charged cell surface (12–14). The main mechanism of delivery to mammalian cells is believed to be endocytosis (15–18), although a membrane fusion mechanism also has been proposed (5, 7, 12).

The effectiveness of gene delivery in vitro does not correspond to the results obtained in in vivo studies (19–20). There is some evidence that serum components inhibit gene transfer by altering the size and shape of CL-DNA complexes (21) and that binding of serum proteins, such as albumin, lipoproteins, and macroglobulin, to CL-oligonucleotide complexes interferes with cellular uptake of the complexes (22). It has also been hypothesized that heparin present in serum could be an inhibitor of gene transfer by releasing the DNA from the CL (22, 23). Moreover, it has been suggested that gene delivery by synthetic CL could be limited by activation of the complement system (22, 24).

Most components in serum with the potential of interacting with gene therapy vehicles are produced by hepatocytes. However, in vivo studies of CL-mediated gene transfer have shown that there are considerable variations in transfection efficiency between different tissues (25–26), suggesting that tissue-specific factors are important regulators. A better understanding of the nature of the molecules that can potentially interfere with CL-DNA complex-based gene delivery may give rise to new strategies for efficient transfection of specific tissues and cell types in vivo. Molecules that are potential inhibitors of CL-mediated gene transfer include proteoglycans (PGs). Their glycosaminoglycan side-chains consist of repetitive disaccharide units with a wide variety of substitutions, which results in a substantial structural heterogeneity (27). The large number of carboxyl and sulfate groups give them a greater linear charge density than DNA (27). PGs are fundamental components of the pericellular matrix, basement membranes, and other extracellular matrices (28–31). Cell surface-associated PGs participate in the regulation of cellular proliferation, differentiation, and migration via specific interactions with various growth factors (30–34).

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† The abbreviations used are: CL, cationic lipid; AMAC, 2-aminoacridone; CM, cell culture medium; CS, chondroitin sulfate; DOGS, di-octadecylamidoglycolyl-spermine; DOPE, dioleoyl phosphatidylethanolamine; DS, dermatan sulfate; HFL-1, human embryonic lung fibroblast; HS, heparan sulfate; NEM, N-ethylmaleimide; PG, proteoglycan; MEM, minimum essential medium; PBS, phosphate-buffered saline.
The objective of this study was to identify secreted molecules that may have diverse negative effects on CL-mediated gene transfer. We present evidence that CL-mediated gene transfer is inhibited by secreted cellular proteins, in particular PGs. PGs form complexes with CLs, leading to the release of DNA and intracellular accumulation of CL-PG complexes. This results in inhibition of DNA uptake (2–4-fold) and a disproportionate inhibition of reporter gene expression (100–1000-fold).

It is also shown that the effects exerted by PGs on CL-mediated gene transfer are mediated by the glycosaminoglycan portion of the PGs, i.e. chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS). Protease sulfate, a polybasic peptide that neutralizes the anticoagulant activity of glycosaminoglycans (35), abrogated the inhibitory effects exerted by PGs. Finally, by fluorescence microscopy it was shown that glycosaminoglycans, internalized by a CL-dependent route, accumulate in the cell nuclei.

**EXPERIMENTAL PROCEDURES**

**Materials—**LipofectAMINE reagent (Life Technologies, Inc.) is a 3:1 (w/v) liposome formulation of the lipopolyamine 2,3-dioleyloxy-N-[3-(trimethoxysilyl)propyl]diethylenetriamine (DOGS) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE). Transfectam reagent, consisting of dioctadecylamidoglycyl-spermine (DOGS) and a luciferase assay kit were obtained from Promega. The PhoKins pyralis luciferase encoding plasmid pGL3 under the control of the SV40 promoter/enhancer, was kindly provided by Dr. Å. Oldberg (Lund University, Lund, Sweden). Plasmid DNA was grown using standard techniques and purified using Qiagen columns (Qiagen Inc.). The rhodamine/luciferase plasmid was purchased from Gene Therapy Systems Inc. 3000 Ci/mmol [32P]dCTP, 1310 Ci/mmol Na235SO4, and a nick translation kit were from Amersham Pharmacia. Microspin S-200 HR columns, Superose 6 HR 10/30, and Mono Q HR 5/5 were from Amersham Pharmacia Biotech. DE 35 DEAE-cellulose was from Whatman, and Centriplus 30 concentrators were from Amicon Inc. Ndihiodum bromide (EtBr) and proteamine sulfate were from Sigma. Microfluor multwell plates were from In Vitro AB. Chondroitin ABC was from Seikagaku Inc. [35S]sulfate and [3H]leucine radioactivity was determined by liquid scintillation counting using ReadySafe scintillation mixture (Beckman) and a Wallac Rack Beta counter (Amersham Pharmacia Biotech). Cells from parallel cultures were counted in a Bürker chamber, and cell-associated DNA was expressed as ng/106 cells.

**Ethidium Bromide Intercalation Assay—**Plasmid DNA (20 µg/ml) was preincubated with EtBr (20 µg/ml) either in MEM or CM (prepared as described above) in a 96-well Microfluor plate. The indicated amount of DOSPA/DOPE or DOGS was added to give a final volume of 100 µl. EtBr fluorescence was monitored at 610 nm, and cell-associated DNA was quantified by liquid scintillation counting.

**Luciferase Gene Expression Assay—**HFL-1 and A 549 cells were plated in 24-well plates at 1 × 104 cells/well in 0.5 ml of growth medium. After 24 h, the medium was aspirated and replaced with 0.5 ml of fresh MEM for 24 h. At the end of the 4-h incubation at 37 °C in 5% CO2, the medium was aspirated, and cells were transferred to an ice bath and washed with ice-cold MEM, and then detached by trypsin (0.05 µg/ml) treatment, followed by two washings with MEM. Luciferase expression was quantified in 5 µl of the cell lysates supernatant, using a luciferase assay kit. Light emission was measured by integration over 30 s at 25 °C using an EG & G Berthold luminometer. Luciferase activity was normalized to the protein content of each sample, determined with the Pierce BCA protein assay.

**Labeling of Plasmid DNA with [32P]dCTP—**Two µg of plasmid DNA pGL3 was labeled with [32P]dCTP 200 Ci/mmol using the chloroform method. Labeling was followed by precipitation using trichloroacetic acid and washed with ice-cold 70% ethanol. Each preparation was diluted to 30 µg/ml DNA in 0.1 M NaCl.

**Labeling of Plasmid DNA with [35S]sulfate—**Plasmid DNA was labeled with [35S]sulfate and then purified using Microspin S-200 HR columns according to the manufacturer’s instructions. After precipitation with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% EtOH.

**Preparation of Radiolabeled, Conditioned Cell Culture Medium—**Confluent monolayers of HFL-1 or a 549 cells in 25-cm2 culture flasks (approximately 1.5 × 107 cells) were labeled with Na32SO4 (50 µCi/ml) and [3H]thymidine (20 µCi/ml) in 4 ml of fresh MEM for 24 h. The medium was aspirated, and cells were extensively washed with MEM to remove free radioisotope. This was followed by another incubation in 4 ml MEM for 24 h. The cell culture medium (CM) was recovered by trypsinization, followed by centrifugation, followed by two washings with MEM. It is also shown that the effects exerted by PGs on CL-mediated gene transfer are mediated by the glycosaminoglycan portion of the PGs, i.e. chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS). Protease sulfate, a polybasic peptide that neutralizes the anticoagulant activity of glycosaminoglycans (35), abrogated the inhibitory effects exerted by PGs. Finally, by fluorescence microscopy it was shown that glycosaminoglycans, internalized by a CL-dependent route, accumulate in the cell nuclei.

**Cells and Media—**Human embryonic lung fibroblasts (HFL-1, CCL-153) and human lung carcinoma cells (A 549, CCL-185) were obtained from the American Type Culture Collection. Monolayer cultures were maintained on plastic in Eagle’s MEM (Life Technologies) supplemented with 15% FCS. 4.2.2.8) were purchased from Seikagaku Inc. [32P]DNA plasmid bands were detected using Molecular Probes. [32P]DNA plasmid bands were detected using Molecular Probes. [32P]DNA plasmid bands were detected using Molecular Probes. [32P]DNA plasmid bands were detected using Molecular Probes.
tion with 1.3 volumes of 7 M urea, 10 mM Tris, pH 7.5, containing 0.1% (v/v) Triton X-100 and 10 mM NEM followed by chromatography on DEAE-cellulose columns (1 ml) equilibrated in the same urea buffer as above. After sample application, the columns were washed with 10 bed volumes of the urea buffer and 10 volumes of 50 mM Tris-HCl, pH 7.5, followed by elution with 3 bed volumes of 4 M guanidinium chloride, 50 mM NaOAc, pH 5.8, 0.2% (v/v) Triton X-100, 10 mM NEM, and 5 µg/ml bovine serum albumin. Eluted material was recovered by precipitation with 8 volumes of ethanol. Samples from procedure 1 were dissolved in 0.4 ml of the 4 M guanidinium chloride-containing elution buffer (without bovine serum albumin) and chromatographed on Superose 6 HR 10/30 (flow rate, 0.4 ml/min), equilibrated in the same buffer. One-min fractions were analyzed for 35S or 3H radioactivity by scintillation counting. Pooled material was concentrated in Centrifil 30 concentrators, recovered by ethanol precipitation, and then applied to a Mono Q HR 5/5 column equilibrated in 7 M urea, 10 mM Tris, pH 7.5, containing 0.1% Triton X-100, pH 8.0 (starting buffer). After a 5-ml wash with starting buffer, the column was eluted with a linear gradient of 0.3–1.2 M NaCl in the same buffer over 60 min. The flow rate was 0.5 ml/min, and 0.5 ml fractions were collected. Aliquots of the collected fractions were analyzed by scintillation counting and pooled as indicated for further analysis.

Enzymatic Degradation—Material collected from ion-exchange chromatography on Mono Q was recovered by ethanol precipitation and dissolved in the appropriate buffer. Digestions with chondroitin ABC lyase (50 milliunits/ml) were performed in 200 µl of 10 mM Tris- HCl, pH 7.3, 10 mM NaEDTA, 10 mM NEM at 37 °C for 10 h. Digestions with HS lyase (50 milliunits/ml) were performed in 200 µl of 3 mM Ca(OAc)2, 10 mM Hepes-NaOH, pH 7.0, 10 mM NEM at 37 °C for 10 h. Digested samples were lyophilized, dissolved in 0.5 ml of the starting buffer, and reapplied to the Mono Q column for linear gradient elution as described above.

Fluorescence Microscopy—HFL-1 cells were seeded at a density of 5 x 104 cells/well in 24-well plates in growth medium. After 24 h, cells were rinsed with MEM and then supplemented with 1 µg/ml of AMAC-HS with or without 8 µg/ml DOSPA/DOPE in MEM. At the end of the 4 h incubation at 37 °C, medium was aspirated, and cells were trypsinized, suspended in growth medium, centrifuged, resuspended in growth medium, and replated on 4-well coverslips. After an additional incubation period of 24 h, cells were washed three times with PBS and fixed in 2% paraformaldehyde in PBS for 30 min, followed by extensive rinsing in PBS and distilled water. Cells were then viewed using a Nikon Diaphot 300 microscope and a Bio-Rad MRC-1024 confocal laser scanning microscope system. The optics used to separate fluorescence signal from noise was a dichroic mirror (562DCLP) and a band-pass filter (D540/30) from Chroma Technology, Inc. The light source used was a Kr+-laser (Coherent Innova 300) supplying 2-mW, 413-nm continuous light. The images were digitized and transferred to a computer workstation for merging, annotation, and printing.

The following procedure was performed to assess the integrity of AMAC-HS isolated from cells: 1.5 x 106 cells were seeded in a 25-cm2 cell culture flask and grown for 24 h. Cells were then incubated with HS-AMAC (100 µg) and CL (800 µg) as described above. After the second incubation period of 24 h, cells were detached by scraping in PBS, centrifuged, and then lysed in the cell extraction buffer. The cell lysate was then passed through a DEAE-cellulose column (1 ml) as described above, in order to separate free AMAC from intact AMAC-HS. Eluted AMAC-HS was recovered by precipitation with ethanol and then dissolved in 200 µl of distilled water. The recovered material from cell cultures and standard solutions of AMAC-HS were then analyzed by fluorescence spectroscopy using a Perkin-Elmer LS-5B spectrometer (excitation wavelength of 430 nm) and a Perkin-Elmer plate reader (emission wavelength of 520 nm).

RESULTS
Inhibition of Cationic Lipid-mediated Gene Transfer by Conditioned Cell Medium Components—We examined the influence of secreted cell molecules on CL-mediated gene transfer in two different types of cells: HFL-1 (human lung fibroblasts) and A 549 (a human lung carcinoma cell line). The CL formulations used were DOSPER/DOPE and DOGS, which are commercially available under the trade names LipofectAMINE and Transfectam, respectively. They are both lipospermines, carrying spermine, a natural counterion of DNA in vivo, as the DNA-binding group. The former transfection reagent also contains the neutral colipid DOPE. Throughout this study, these transfection reagents behaved very similarly.

CL-mediated transfection was inhibited by CM in both HFL-1 and A 549 cells (Table I). At the highest lipid concentration used (16 µg/ml), transfection was inhibited by 99.5 and 99.9% in HFL-1 and A 549 cells, respectively. There was a substantial difference in transfection efficiency between the two cell lines (approximately 37-fold higher in A 549 compared with HFL-1). Conditioned CM obtained after shorter incubation periods was less inhibitory, but CM isolated already after 8 h significantly reduced gene expression. CM obtained after a 24 h-incubation was chosen for further studies. In a control experiment, plasmid DNA was incubated either in MEM or CM for 24 h at 37 °C and then analyzed for integrity by agarose gel electrophoresis. There was no difference in the recovery of intact plasmid (approximately 87%), suggesting that the inhibition of gene expression by CM was not due to increased degradation of the reporter gene plasmid. Moreover, preexposure of HFL-1 cells to CM for 4 h, prior to the addition of CL-DNA complexes, did not affect gene transfer efficiency. However, preexposure of cells for 4 h to CM supplemented with 16 µg/ml CL inhibited gene expression by approximately 85% compared with cells preexposed to MEM supplemented with CL (results not shown).

We then investigated whether the decrease in gene expression caused by CM originated from a reduced uptake of CL-DNA complexes. As shown in Fig. 1, there was a significant reduction of the amount of internalized DNA plasmid when the experiments were performed in CM as compared with MEM. The decrease in DNA uptake (approximately 60% at 16 µg/ml CL) was not proportional to the inhibition of gene expression. Cells preexposed to CM supplemented with 16 µg/ml CL internalized an equal amount of DNA plasmid as compared with

| Table I | Influence of CM components on CL-mediated transfection efficiency |
|---------|---------------------------------------------------------------|
| CL      | HFL-1              | Luciferase activity | A 549              |
| µg/ml   | Control           | CM                | Control           | CM                |
| 0       | 0.01 ± 0.002      | 0.09 ± 0.002      | 0.2 ± 0.0005      | 0.06 ± 0.002      |
| 2       | 0.03 ± 0.003      | 0.5 ± 0.03        | 55 ± 1.0          | 0.4 ± 0.03        |
| 8       | 47 ± 1.1          | 1900 ± 58        | 17900 ± 26        | 0.2 ± 0.01        |
| 16      | 389 ± 27          | 3300 ± 76        | 3300 ± 76        | 3.6 ± 0.03        |
cells preexposed to MEM supplemented with CL (results not shown).

It is worth noting that the difference in luciferase activity between the cell lines did not correspond to the difference in DNA uptake (approximately 1.3 times higher in HFL-1 cells compared with A 549 cells at a CL concentration of 16 μg/ml).

**Effect of Cell Medium Components on Cationic Lipid-DNA Complex Formation**—One possible explanation for the inhibitory activity of CM on the uptake of CL-DNA complexes is that the complexes dissociate upon interaction with CM components. We therefore studied the stability of CL-DNA complexes in CM using an EtBr intercalation assay. When EtBr is mixed with double-helical DNA in solution, it intercalates between the base pairs, emitting an intense fluorescence signal at 610 nm when excited at 500 nm. It has been reported that the addition of CL results in an immediate and substantial (approximately 90%) decrease of the fluorescence signal, as a result of the displacement of EtBr from the DNA (38). In the present study, a dose-dependent inhibition of the fluorescence signal was obtained with DOSPER/DOPE (Fig. 2, □). When the same experiment was performed in CM, a significant amount of the EtBr fluorescence was recovered, suggesting a reversal of CL-DNA complex formation and increased accessibility of the EtBr intercalation site (Fig. 2, ◊). The detection limit of the EtBr intercalation assay (20 μg/ml DNA) did not permit the use of the same concentration of CL and DNA as in the gene expression and DNA uptake experiments (1 μg/ml DNA, see Table I and Fig. 1).

**Cationic Lipid-dependent Cellular Uptake of Cell Medium Components**—Altogether, the results described above suggest that CM inhibits CL-mediated uptake of DNA plasmid by interfering with the formation of CL-DNA complexes, resulting in reduced gene expression. However, the decrease in DNA uptake was not proportional to the inhibition of gene expression. Moreover, preexposure of cells to conditioned CM supplemented with CL significantly reduced gene expression, although subsequent uptake of DNA in MEM was equal as compared with control (cells preexposed to MEM supplemented with CL).

In order to gain more insight into the mechanism of these effects, CM that had been metabolically labeled with [35S]sulfate and [3H]leucine was mixed with CL and then added to unlabeled HFL-1 and A 549 cells, respectively (Fig. 3). DNA plasmid (1 μg/ml) was included in the respective media in order to mimic the experimental conditions in previous experiments. Control cell cultures (no CL added) internalized a significant amount of [3H]-labeled protein, whereas the uptake of [35S]sulfate-labeled compounds was very limited in both cell types. Addition of CL caused a dose-dependent increase in the uptake of both [3H]leucine- and [35S]sulfate-labeled material (up to 8 and 16 μg/ml DOSPER/DOPE in HFL-1 and A 549 cells, respectively). In particular, internalization of components labeled with [35S]sulfate was favored by the presence of CL. At the CL concentrations indicated above, approximately 85 and 79% of total [35S]sulfate-labeled material in CM from HFL-1 and A 549 cells, respectively, was associated with the cells. These results suggest that CL-DNA complex formation was inhibited primarily by [35S]sulfate-labeled components in CM and that these components were internalized as a complex with the CL.

**Identification of Internalized Cell Medium Components and Their Effects on Gene Transfer**—To study the nature of the internalized CM components, detergent extracts of cells incubated with radiolabeled CM either in the absence or in the presence of various amounts of CL were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, CL caused a dose-dependent increase in the uptake of [35S]methionine-labeled proteins (corresponding to the results presented in Fig. 3), in particular high molecular weight components.

In a similar set of experiments, [35S]sulfate-labeled CM components, taken up by the cells in the presence of CL, were recovered by ion exchange chromatography on DEAE-cellulose (approximately 90% recovery of the starting material) and then analyzed by gel filtration chromatography, revealing the existence of one major component eluting with the void volume and one minor more retarded component (Fig. 5A). These components were further studied by ion-exchange chromatography on Mono Q eluted with a linear gradient (Fig. 5B). This procedure separated the material into two distinct populations, eluting at approximately 0.83 (pool I) and 1.0 M NaCl (pool II). A series of degradation experiments were then performed to specifically identify the sulfated, polyanionic macromolecules. The differential susceptibility of the material in pool I and pool II to HS lyase and chondroitin ABC lyase, showed that pool I consisted entirely of HSPG (Fig. 5C), whereas pool II contained a mixture of HSPG and CS/DSPG (Fig. 5D). Treatment of the material in
and \(^{3}H\)leucine (20 \(\mu\)Ci/ml) and \(^{35}S\)sulfate (50 \(\mu\)Ci/ml) as described under "Experimental Procedures." One \(\mu\)g/ml DNA plasmid and 0, 4, 8, or 16 \(\mu\)g/ml CL, respectively, were included in the incubation media. After incubation, for 4 h at 37 °C the amount of cell-associated \(^{35}S\)sulfate \((\text{C})\) was measured by scintillation counting. Data points represent the mean ± S.E. \((\text{error bars}) \(n = 6\).

FIG. 3. CL-mediated cellular uptake of CM components. HFL-1 (A) and A 549 (B) cells were plated in 24-well dishes at \(1 \times 10^{4}\) cells/well for 24 h and then washed twice with MEM, followed by incubation with fresh conditioned CM from cells labeled with \(^{35}S\)sulfate (50 \(\mu\)Ci/ml) and \(^{3}H\)leucine (20 \(\mu\)Ci/ml) for 4 h at 37 °C the medium was removed followed by extensive washing with PBS. Proteins isolated from a detergent extract of the cells were dissolved in SDS buffer and run on SDS-polyacrylamide gradient gel electrophoresis. The migration positions of the protein standards are indicated on the left.

FIG. 4. SDS-polyacrylamide gel electrophoresis of internalized CM proteins. HFL-1 cell cultures in 25-cm\(^2\) flasks were incubated with \(^{35}S\)methionine-labeled CM either in the absence \((\text{lane 1})\) or in the presence of 8 \(\mu\)g/ml \((\text{lane 2})\) or 16 \(\mu\)g/ml \((\text{lane 3})\) CL. After incubation for 4 h at 37 °C the medium was removed followed by extensive washing with PBS. Proteins isolated from a detergent extract of the cells were dissolved in SDS buffer and run on SDS-polyacrylamide gradient gel electrophoresis. The migration positions of the protein standards are indicated on the left.

pool II with both lyases resulted in a complete degradation (data not shown). By gel filtration chromatography on Superose 6, it was confirmed that the \(^{35}S\)sulfate-labeled CM compounds consisted of intact PGs rather than free glycosaminoglycan chains, as the latter (released from the protein core by alkali treatment) exhibited a significantly more retarded elution position on Superose 6 (data not shown).

When the experiments presented in Fig. 5 were conducted with CM labeled with \(^{3}H\)GlCN, the same results were obtained, indicating that other glycoproteins and hyaluronic acid were not internalized as a complex with CL, under the conditions described here (data not shown).

PGs purified from CM, as shown in Fig. 5, were tested for inhibitory activity on CL-mediated gene transfer. The inhibition of gene expression by PGs almost mirrored that of CM as a whole, indicating that PG was the major inhibitory component in CM (Table II). Pretreatment of PG with HS lyase or ABC lyase diminished the inhibitory activity by approximately 75 and 25%, respectively, suggesting that HSPG played a more important role than CS/DSPG. Moreover, exogenous addition of either HS or DS at concentrations as low as 5 \(\mu\)g/ml completely abolished CL-mediated gene transfer, suggesting that the glycosaminoglycan portion of the PG was mediating the inhibition of gene transfer. It was not possible to digest the PG with the corresponding lyases directly in CM.

Pretreatment of Cell Culture Medium with Protamine Sulfate Restores Transfection Efficiency—Protamine sulfate is a United States Food and Drug Administration-approved compound with a known neutralizing effect on the anticoagulant activity of heparin and DS in vivo \((35)\), due to its polycationic nature. DNA plasmid uptake and gene expression were measured either in CM or MEM, pretreated with increasing concentrations of protamine sulfate. An optimal ratio of DNA to DOSPER/DOPE, as determined in the experiments described above (see Table I and Fig. 1) was used. As shown in Fig. 6A, protamine sulfate specifically potentiated DNA uptake in cells incubated with CM. At the highest protamine sulfate concentration used \((100 \mu\)g/ml), DNA uptake was almost 3-fold greater as compared with the control \((\text{no protamine sulfate added})\). Addition of protamine sulfate to MEM only had a limited effect on DNA uptake by HFL-1 cells. Accordingly, pretreatment of CM with 10 \(\mu\)g/ml protamine sulfate restored gene expression to the same level as in MEM \((\text{Fig. 6B})\). Furthermore, pretreatment of CM with protamine sulfate reduced the CL-mediated intracellular accumulation of \(^{35}S\)PG (data not shown).

Nuclear Accumulation of Glycosaminoglycans—Confocal microscopy studies with rhodamine-labeled DNA plasmid confirmed the results presented above. The uptake of DNA was significantly reduced by CM components and exogenous glycosaminoglycans. However, we were not able to observe an altered intracellular distribution of rhodamine-DNA as compared with cells incubated with MEM \((\text{results not shown})\).

We then studied the effect of CL on the cellular uptake of DS...
TABLE II

Effects on CL-mediated transfection of CM, PG isolated from CM, enzymatically treated PG isolated from CM, and glycosaminoglycans

HFL-1 cell cultures were incubated for 4 h with 16 μg/ml CL and 1 μg/ml DNA plasmid in their respective media, as indicated. Then cells were incubated for another 48 h in growth medium, followed by a luciferase assay. PGs isolated from CM were diluted in an equivalent volume of MEM to obtain the original concentration of PG found in CM. The concentration of DS and HS was 5 μg/ml. For details, see legend to Fig. 5. Luciferase activity is expressed as mean ± S.E. (n = 6).

| Medium          | Luciferase activity | 10^3 × relative light units/μg of protein |
|-----------------|---------------------|------------------------------------------|
| MEM             | 85 ± 2.0            |                                          |
| CM              | 1.3 ± 0.06          |                                          |
| MEM + PG        | 16 ± 1.2            |                                          |
| MEM + PG + E7   | 71 ± 0.9            |                                          |
| MEM + PG + ABC lyase | 30 ± 0.6       |                                          |
| MEM + PG + HS lyase | 57 ± 0.7       |                                          |
| MEM + DS        | 0.2 ± 0.01          |                                          |
| MEM + HS        | 0.3 ± 0.02          |                                          |

* PG treated with HS lyase and chondroitin ABC lyase.

and HS, using 125I-labeled polysaccharide chains. Fig. 7A shows that the addition of CL increased the uptake of both HS and DS chains in a dose-dependent manner, corresponding to the results presented in Fig. 3. As the nucleus is the intracellular target for transduced DNA, it was of interest to study the subcellular localization of internalized glycosaminoglycan chains. HS, end-labeled with the fluorophore AMAC, was added to cells either with or without CL (8 μg/ml) for 4 h, followed by an incubation period of 24 h in growth medium. In the absence of CL, the amount of intracellular HS was low with a diffuse extranuclear localization (Fig. 7B). On the contrary, in the presence of CL, there was a substantial nuclear accumulation of HS and a relatively low amount of HS in the extranuclear compartment (Fig. 7C). Altogether, CL caused an increase in the amount of internalized glycosaminoglycans and nuclear accumulation of HS. This pattern was obtained after a growth period of approximately 24 h. Analysis of cells exposed to HS-CL complexes after shorter growth periods showed a less pronounced nuclear accumulation and a stronger extranuclear fluorescence (results not shown). Intracellular AMAC-HS was assessed by integrity by reisolation of the material from cell cultures as described under “Experimental Procedures,” showing that all of the AMAC-HS remained intact (results not shown).

**DISCUSSION**

This study provides the first evidence that molecules secreted by cells are inhibitory to CL-mediated DNA uptake and gene expression. Our results indicate a dramatic inhibitory effect on gene expression caused by intracellular accumulation of CM components, in particular sulfated PGs. PGs isolated from CM were found to inhibit CL-mediated gene transfer almost to the same degree as whole CM, and this effect could be reversed by enzymatic treatment of PGs with glycosaminoglycan lyases. Exogenous glycosaminoglycans also abolished CL-mediated gene transfer. CM components interfered with CL-DNA complex formation, resulting in reduced cellular uptake of DNA plasmids with a concomitant increase in the uptake of sulfated PGs. Low amounts of CM-derived PGs were internalized in the absence of CL. Cells preexposed to CM supplemented with CL exhibited a substantial decrease in gene expression, although DNA uptake activity was unaffected, suggesting that intracellular accumulation of CM components per se results in inhibition of gene expression. Preexposure of cells to CM without CL affected neither gene expression nor DNA uptake. Zahner et al. (16) presented evidence that the most important barriers to CL-mediated transfection are the intracellular dissociation of DNA from CL and the movement of DNA from endosomes into the nucleus, respectively. One possible explanation of our results is that intracellular accumulation of
PGs/glycosaminoglycans interferes with these processes.

It has been demonstrated by fluorescence resonance energy transfer and gel shift assay studies that heparin displaces oligonucleotides from another type of CL (22). However, under physiological conditions in vivo, it is likely that CS/DS and HSPG/glycosaminoglycans, rather than heparin (which is mainly confined to the mast cell granules), will act as barri ers to CL-mediated gene transfer. Spermine, the cationic group of DOSPA and DOGS, has been shown to interact with DS (39) and HS (40) with similar or higher affinity, respectively, than with DNA.

Fibroblasts have a major influence on the production and assembly of the extracellular matrix, which makes HFL-1 cells suitable for the aim of this study. As a comparison, we selected a cancer cell line (A 549) from the same source. The difference in gene transfer efficiency between HFL-1 and A 549 cells was probably due to the superior ability of A 549 cells to replicate pGL3 plasmid DNA, which is in accordance with a previous report, in which human and murine melanoma cells internalized equivalent amounts of CL-DNA complexes, yet the level of transgene expression differed considerably between the cell lines studied (41).

By confocal microscopy studies, we also demonstrated that exogenous glycosaminoglycans, such as HS, accumulate in the cell nuclei, a process that seems to be dependent on CL. It has been suggested that nuclear HS may play a role in regulating nuclear activities (42). In the same report, exogenous HS was reisolated from hepatocytes, in which approximately 10% of the internalized material was confined to the nuclei. In our study, an insignificant amount of HS was internalized in the absence of CL, with no localization to the cell nuclei, which partly may be explained by the relatively short period of incubation. It is noteworthy that HS variants exhibiting strong binding to spermine inhibit the proliferation of human lung fibroblasts by up to 63% (40).

A number of investigations have been directed at studying the production of PGs in fibroblasts, showing that the major forms secreted to the extracellular matrix are the CS/DSPGs decorin and versican. Fibroblasts also secrete a HSPG with a 250–400-kDa protein, probably perlecan (37, 43, 44). Conditioned media may also contain shed cell-surface HSPGs, such as syndecan, glypican, or betaglycan. Syndecans can be hybrid PGs/glycosaminoglycans, rather than heparin (which is essential for the internalization of CL-DNA complexes, or 2) enzymatic release of glycosaminoglycan chains in the tissues, preventing the CL-DNA complexes from reaching their target cells. Either of these mechanisms indicates a role for PGs in CL-mediated gene transfer in vivo.

The use of synthetic polycationic lipids for the delivery of negatively charged nucleic acids has been proven to be an important alternative to viral gene transfer. Although in vivo gene delivery mediated by CL does occur (9–11), a synthetic compound relying on electrostatic interactions with the gene to be delivered is likely to exhibit unwanted interactions with other negatively charged components residing in the extracellular matrix and in the serum. Potential strategies to overcome the PG-mediated inhibition of gene transfer in vivo are suggested by our study. Pretreatment with the polycationic peptide protamine sulfate neutralized the inhibition of gene transfer by CM. Because protamine sulfate has been proven to be nontoxic and only weakly immunogenic in humans, this compound may be useful for gene therapy in vivo. Indeed, the addition of protamine sulfate to CL-DNA complexes has been shown to increase gene transfer efficiency in vivo (48). Preinjection of polycationic compounds such as protamine sulfate could potentially block the inhibitory action of PGs and other polyanionic compounds in serum and the extracellular compartment.

In conclusion, our results support the view that the lack of specificity of this class of gene transfer vehicles is a major hindrance to efficient and safe administration. Future studies should be aimed at developing gene delivery vehicles based on structures with higher binding specificities for DNA. Another possibility would be to manipulate the production and distribution of PGs and other components capable of interfering with CL-mediated gene transfer.

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