Protective role for proteoglycans against cationic lipid cytotoxicity allowing optimal transfection efficiency *in vitro*

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A dependence on proteoglycans for cationic lipid-mediated gene transfer has been suggested in previous studies [Mislick and Baldeschwieler (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 12349–12354; Mounkes, Zhong, Cipres-Palacin, Heath and Debs (1998) J. Biol. Chem. **273**, 26164–26170]. We have evaluated the mechanism of proteoglycan involvement in cationic lipid-mediated gene transfer. DNA plasmid uptake and gene expression were studied in wild-type Chinese hamster ovary (CHO) cells (CHO-K1), heparan sulphate-deficient CHO cells (pgsD-677) and proteoglycan-deficient CHO cells (pgsB-618). At an optimal ratio of cationic lipid to DNA, a substantial decrease in reporter gene expression was observed in proteoglycan-deficient cells compared with that in heparan sulphate-deficient and wild-type cells. However, there were no differences in reporter gene expression between the cell lines when transfected by electroporation. Moreover, all cell lines exhibited equal cationic-lipid–DNA complex uptake activities, as assessed by the measurement of intracellular ^{32}P-labelled and rhodamine-labelled DNA plasmid. An analysis of reflected-light images of wild-type and proteoglycan-deficient cells suggested that cationic lipids were preferentially toxic to proteoglycan-deficient cells. Cell-growth assays confirmed this, showing that cationic lipids exhibited a greater anti-proliferative activity in proteoglycan-deficient cells and in chlorate-treated wild-type cells than in the other cell lines. The growth-inhibitory effect of cationic lipids was abrogated by the addition of exogenous sulphated glycosaminoglycans. We conclude that the glycosaminoglycan part of proteoglycans serves a protective role against cationic lipid cytotoxicity, allowing optimal transfection efficiency *in vitro*.

Key words: gene therapy, glycosaminoglycan, liposomes, polyamines.

INTRODUCTION

Substantial efforts have been focused on the development of gene transfer vehicles that could be used for gene therapy *in vivo* [1–4]. Among them are polybasic peptides and cationic lipids (CLs) respectively, which act by forming stable complexes with DNA via electrostatic forces [5,6]. Under optimum conditions the CL–DNA complexes exhibit a positive net charge that is a prerequisite for efficient uptake into the target cells [5]. The molecules on the cell surface that are responsible for the interaction with and uptake of CL–DNA complexes have not been thoroughly defined. The mechanism of passage over the cell membrane is believed to be endocytosis [7–10], although the lipids used to form the complexes are known to adopt structures that can engender direct fusion with plasma membranes [5,11,12].

Owing to their polyanionic nature and association with the cell membrane, proteoglycans (PGs) are potential candidates for the binding and uptake of positively charged compounds such as polybasic peptides and CLs. PGs are a large group of macromolecules consisting of glycosaminoglycan chains, e.g. chondroitin sulphate (CS), dermatan sulphate (DS) and heparan sulphate (HS), that are covalently attached to a protein core [13]. PGs are fundamental components at cell surfaces and in the extracellular matrix [13–16]. They are known to regulate the activity of several cytokines, enzymes and growth factors such as transforming growth factor β [17,18], lipoprotein lipase [19,20] and basic fibroblast growth factor [21,22] respectively. Several viruses bind to and infect cells via interactions with cell-surface PG [23,24].

It has been suggested that transfection by CLs is dependent on cell-surface PG [25]. Reporter gene expression was decreased by up to 80-fold in Chinese hamster ovary (CHO) cells deficient in PG synthesis compared with wild-type cells, at an optimum ratio of CL to DNA. Moreover, HSPGs were specifically implicated in polylysine-mediated transfection. In another study [26] it was suggested that PGs are significant in CL-mediated gene delivery and gene expression both *in vitro* and *in vivo*. Raji cells, deficient in PG synthesis, were virtually untransfected by CL–DNA complexes, whereas stable expression of the HSPG syndecan-1 in the same type of cell resulted in efficient transfection.

A better understanding of the functional molecules involved in gene delivery is a prerequisite for the development of more efficient and specific gene transfer vehicles *in vivo*. This study provides evidence that (1) HSPGs and/or CS/DSPGs are required for optimal CL-mediated transfection *in vitro*, (2) cell-surface PGs are not required for the internalization of CL–DNA complexes, and (3) cells deficient in sulphated glycosaminoglycans, including mutant cells and chlorate-treated wild-type cells, exhibit increased sensitivity to CL cytotoxicity.

EXPERIMENTAL

Materials

The *Photinus pyralis* luciferase-encoding plasmid pGL3 under the control of the SV40 promoter/enhancer, was a gift from Dr. Å. Oldberg (Lund University, Lund, Sweden). Plasmid DNA was grown by using standard techniques and purified with the

Abbreviations used: CHO, Chinese hamster ovary; CL, cationic lipid; CS, chondroitin sulphate; DOPE, dioleoyl phosphatidylethanolamine; DOSPA, 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium; DS, dermatan sulphate; HS, heparan sulphate; PG, proteoglycan.

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use of Qiagen columns. The rhodamine/luciferase plasmid was purchased from Gene Therapy Systems. Lipofectamine reagent (Life Technologies) is a 3:1 (w/w) liposome formulation of the lipopolyamine 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propanaminium (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE). A luciferase assay kit was obtained from Promega (Sweden); [^{32}P]dCTP (3000 Ci/mmol), $Na_2{}^{35}SO_4$ (1310 Ci/mmol) and a nick translation kit were from Amersham Life Sciences, and Microspin S-200 HR columns were from Pharmacia–LKB. Ethidium bromide was obtained from Sigma Chemicals, and electroporation cuvettes were from BTX. The HS preparation (HS-6) was derived from beef lung as described elsewhere [27]. In brief, heparin by-products were dissolved in 2.1 M NaCl and subjected to stepwise precipitation as cetylpyridinium complexes into subfractions of decreasing charge density. HS-6 (molecular mass 20 kDa) was obtained between 1.2 and 2.1 M NaCl, constituting approx. 28 % of the starting material. The ratios of *N*-sulphate to hexosamine and of total sulphate to hexosamine were 0.72 and 1.63 respectively; iduronic acid constituted 65 % of the uronic acid residues. The DS preparation was the same as that described previously [28].

Cells and media

Wild-type CHO cells (CHO-K1), CHO cells deficient in HSPG biosynthesis (pgsD-677) and CHO cells deficient in PG biosynthesis (pgsB-618) were all obtained from the American Type Culture Collection. Monolayer cultures were maintained on plastic in F-12K nutrient mixture (Life Technologies) supplemented with 10 % (v/v) foetal calf serum (In Vitro AB), 2 mM L-glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin (growth medium) in an incubator under humidified air/CO_2 (19:1) at 37 °C. Cells were checked for mycoplasma by using GEN-PROBE (Skafte & Claesson).

Reporter gene expression assay

CHO cells were seeded in 24-well plates at 10^5 cells per well (approx. 50 % confluence) in 0.5 ml of growth medium 24 h before transfection. To prepare CL–DNA complexes, pGL3 plasmid DNA (1 μg/ml) and the desired amount of DOSPA/DOPE or dioctadecylamidoglycyl-spermine were diluted separately in 100 μl of F-12K medium without serum, then mixed by inversion and incubated for 30 min at room temperature. The DNA–lipid mixture was diluted to a final volume of 250 μl, vortex-mixed and added to the cells, which had been rinsed twice with 0.5 ml of F-12K medium. At the end of the 4 h incubation at 37 °C under air/CO_2 (19:1), the medium was aspirated and replaced by 0.5 ml of growth medium per well. After an additional 48 h of incubation at 37 °C under air/CO_2 (19:1), cells were washed twice with F-12K medium and lysed with 150 μl of cell culture lysis reagent containing 25 mM Tris/HCl, pH 7.8, 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 2 mM dithiothreitol, 10 % (v/v) glycerol and 1 % (v/v) Triton X-100 (Promega) for 10 min at 4 °C, followed by scraping. Luciferase expression was quantified on 5 μl of the cell lysate supernatant by using a luciferase assay kit. Light emission was measured by integration over 10 s at 25 °C with an EG & G Berthold Microlumat LB 96P luminometer (Wallac). As a control, cells were transfected by electroporation by the following procedure: CHO cells were seeded in six-well plates (2×10^5 cells per well) and grown for 24 h, then detached by treatment with trypsin (0.5 mg/ml) in 10 mM KH_2PO_4 (pH 7.5)/0.15 M NaCl (PBS) followed by one wash with 1 ml of PBS. Cells were suspended in 0.7 ml of PBS; 5 or 15 μg/ml DNA plasmid was then added. The cell suspension was transferred to a 4 mm gap cuvette that was placed in a Bio-Rad Gene Pulser, supplying a 0.3 kV, 500 μF pulse. Cell suspensions were added to 24-well plates (0.2 ml per well) and diluted with growth medium (0.8 ml per well); this was followed by a growth period of 48 h with an exchange of medium at 24 h. Luciferase activity was normalized to the protein content of each sample, determined with the Pierce bicinchoninic acid protein assay.

Labelling of plasmid DNA with [^{32}P]dCTP

Plasmid DNA pGL3 (2 μg) was labelled with ^{32}P by nick translation with DNase I (100 pg), DNA polymerase I (5 units), 0.7 nmol of dATP, dGTP and dTTP and 33 pmol of [^{32}P]dCTP for 2 h at 15 °C in 50 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/50 μg/ml BSA/0.1 mM dithiothreitol in a final volume of 110 μl. The reaction was terminated by the addition of 10 μl of 0.2 M EDTA. Labelled DNA plasmid was purified from unincorporated nucleotides on Microspin S-200 HR columns in accordance with the manufacturer's instructions, followed by precipitation with 0.1 vol. of 3 M sodium acetate and 2.5 vol. of 95 % (v/v) ethanol. DNA was recovered by centrifugation at maximum speed (12000 ***g***; 14000 rev./min) in a benchtop centrifuge for 4 min. After washing in 70 % (v/v) ethanol, isolated DNA was assessed for integrity by 0.8 % agarose gel electrophoresis at 70 V in Tris/acetate/EDTA buffer for 45 min. [^{32}P]DNA plasmid bands were detected by staining with ethidium bromide.

Cellular uptake of [^{32}P]DNA

CHO cells were seeded in 24-well plates at 10^5 cells per well in 0.5 ml of growth medium. After 24 h, cells were rinsed twice with 0.5 ml of F-12K medium and then supplemented with 1 μg/ml pGL3 plasmid [^{32}P]DNA (specific radioactivity approx. 10^7 c.p.m./μg) and various amounts of CL, as described above. After 4 h of incubation at 37 °C, cells were kept on ice and washed with ice-cold F-12K. Cells were then detached by treatment with trypsin (0.5 mg/ml) in PBS for 1 min at 20 °C, followed by suspension in 0.5 ml of serum-containing medium. Cells were pelleted by centrifugation followed by two washings with F-12K medium. CL–DNA complexes that were non-specifically attached to the cells were removed by this procedure. Cells were then lysed in 0.5 ml of cell culture lysis reagent (defined above) for 10 min at 20 °C; [^{32}P]DNA radioactivity was determined in 250 μl of the cell lysate by liquid-scintillation counting with Readysafe scintillation cocktail (Beckman) and a Wallac Rack Beta counter (Pharmacia-LKB). Parallel cell cultures were counted in a Bürker chamber and cell-associated DNA was expressed as ng per 10^6 cells.

Fluorescence microscopy

CHO cells were seeded at 5×10^5 cells per well in growth medium. After 24 h, cells were rinsed twice with F-12K medium and then supplemented with 1 μg/ml rhodamine-labelled DNA plasmid and DOSPA/DOPE, as indicated. At the end of the 4 h incubation at 37 °C, medium was aspirated and cells were trypsin-treated, suspended in growth medium, centrifuged, resuspended in growth medium and replated on four-well coverslips. Non-specifically bound rhodamine–DNA–CL complexes were removed with this procedure. After incubation for a further 48 h, cells were washed three times with PBS, fixed in 2 % (w/v) paraformaldehyde in PBS for 30 min, followed by extensive

rinsing in PBS and distilled water. Cells were then viewed with a Nikon Eclipse E800 microscope and a Bio-Rad MRC-1024 confocal laser scanning microscope system with a combination of 488/568/647 PC and 560DRLP filters (Chroma Technology). The light source used was an Ar^+ laser (Bio-Rad) supplying 6 mW of continuous light at 568 nm. Reflected light images were obtained from illumination with continuous light at 488 and 568 nm with the same set-up. The images were digitized and transferred to a workstation for merging, annotation and printing.

Cell-growth assay

Essentially the same procedure was used as that described previously [29]. In brief, CHO cells were seeded in 96-well microplates at a density of 3000 cells per well and left to adhere for 4 h in growth medium; this was followed by a starvation period of 24 h in serum-free medium or in F12 medium supplemented with 25 mM chlorate. The medium was aspirated and cells were rinsed twice with 0.2 ml of F-12K medium without serum. Cells were then incubated for 4 h at 37 °C either in F-12K medium or in F12 medium containing 25 mM chlorate, supplemented with various substances as indicated in the figure legends. Then medium was changed to growth medium and cells were grown for an additional 96 h. Cell-growth was determined from the amount of Crystal Violet adsorbed on the cells as measured in a Multiscan 351 photometer (Labsystems) at 595 nm.

Effect of chlorate on [^{35}S]PG production in wild-type CHO cells

CHO-K1 cells were seeded in six-well plates at 2×10^5 cells per well and grown to subconfluence (approx. 8×10^5 cells per well) in growth medium. Cells were then preincubated for 1 h in sulphate-deficient $MgCl_2$ medium, followed by incubation for a further 24 h either in $MgCl_2$ medium or in sulphate-deficient F12 medium supplemented with 25 mM chlorate. [^{35}S]Sulphate (50 μCi/ml) was also added to the cell cultures. Medium was aspirated followed by extensive washing with ice-cold PBS. Then cells were extracted for 10 min at 4 °C with 1 ml of 2 % (v/v) Triton X-100 in PBS containing 10 mM EDTA, 10 mM *N*-ethylmaleimide and 1 mM di-isopropyl fluorophosphate. Extracts were then diluted with 1.3 vol. of 7 M urea/10 mM Tris/HCl (pH 7.5) containing 0.1 % (v/v) Triton X-100, followed by chromatography on DEAE-cellulose columns (1 ml) equilibrated in the same urea buffer as above. After the application of sample, the columns were washed with 10 bed vol. of the urea buffer and 10 vol. of 50 mM Tris/HCl, pH 7.5, followed by elution with 5 bed vol. of 4 M guanidinium chloride/50 mM sodium acetate (pH 5.8)/0.2 % (v/v) Triton X-100/10 mM *N*-ethylmaleimide/5 μg/ml BSA. The amount of [^{35}S]PG in the eluted fractions was determined by scintillation counting.

RESULTS

PGs are required for optimal CL-mediated transfection efficiency

To investigate the influence of PG on CL-mediated gene transfer, the following cell lines were used: (1) wild-type CHO-K1 cells, which produce mainly HSPG [30], (2) mutant pgsB-618 cells deficient in PG biosynthesis owing to the lack of the common galactosyltransferase I [31], and (3) mutant pgsD-677 cells deficient in HS biosynthesis owing to the lack of both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities, probably combined in a heparan polymerase [30]. It is noteworthy that HS-deficient cells accumulate 3–4-fold more CSPG than wild-type cells [30]. CL-dependent reporter gene expression was determined in each cell line by using a luciferase reporter gene assay. As shown in Table 1, PG-deficient cells exhibited significantly higher expression levels than wild-type and HS-deficient cells up to 2 μg/ml CL. At 4 μg/ml CL, the expression level was almost equal for all the three cell lines studied. However, at higher CL concentrations there was a marked decrease in gene expression in PG-deficient cells, whereas a continuous, dose-dependent increase in expression was observed in wild-type and HS-deficient cells. In a control experiment (Table 2), wild-type and mutant cells were transfected by electroporation. Reporter gene expression levels were similar in both cells, which indicates that there were no differences in gene expression between the two cell lines.

Table 1 CL-mediated gene transfer in wild-type, HS-deficient and PG-deficient CHO cells

Wild-type, HS-deficient and PG-deficient cells were seeded at 10^5 cells per well 24 h before the addition of CL and 1 μg/ml DNA plasmid at ratios from 1:2 to 16:1 (w/w) in serum-free medium. After incubation for 4 h at 37 °C, medium was aspirated; this was followed by incubation for a further 48 h in growth medium. Cells were then subjected to a luciferase assay as described in the Experimental section. Results are means ± S.E.M. ($n = 6$).

	$10^{-3} \times$ Luciferase activity (relative light units)		
CL (μg/ml)	Wild-type	HS-deficient	PG-deficient
0	0.09 ± 0.002	0.09 ± 0.002	0.1 ± 0.005
0.5	0.2 ± 0.003	0.2 ± 0.002	0.7 ± 0.003
1	2.0 ± 0.08	1.0 ± 0.05	34 ± 0.6
2	39 ± 0.7	2.5 ± 0.09	306 ± 2.4
4	5800 ± 51	5300 ± 68	5900 ± 51
8	6600 ± 80	7300 ± 84	3000 ± 57
16	7600 ± 96	7600 ± 91	580 ± 6.9

Table 2 Transfection efficiency by electroporation in wild-type and PG-deficient CHO cells

Wild-type and PG-deficient cells were seeded in six-well plates (2×10^5 cells per well) and grown for 24 h, then detached by treatment with trypsin, followed by the addition of 5 or 15 μg/ml DNA plasmid in PBS. The cell suspensions were electroporated as described in the Experimental section. Cells were then seeded in 24-well plates, followed by a growth period of 48 h. Cell lysates were analysed by a luciferase assay; results are presented as means ± S.E.M. ($n = 6$).

	$10^{-3} \times$ Luciferase activity (relative light units/μg of protein)	
DNA plasmid (μg/ml)	Wild-type	PG-deficient
5	930 ± 19	900 ± 21
15	1900 ± 21	2000 ± 38

Efficient uptake of CL–DNA complexes by PG-deficient cells

We next investigated whether the differences in reporter gene expression between PG-deficient cells and the other cell lines originated from differences in the uptake of CL–DNA complexes. The uptake of [^{32}P]DNA plasmid by wild-type, HS-deficient and PG-deficient CHO cells was studied (Figure 1). At 2 μg/ml CL, DNA uptake was statistically different ($P < 0.01$) between the cell lines, with approx. 2- and 3.5-fold greater uptake in PG-deficient cells than in wild-type and HS-deficient cells respectively.

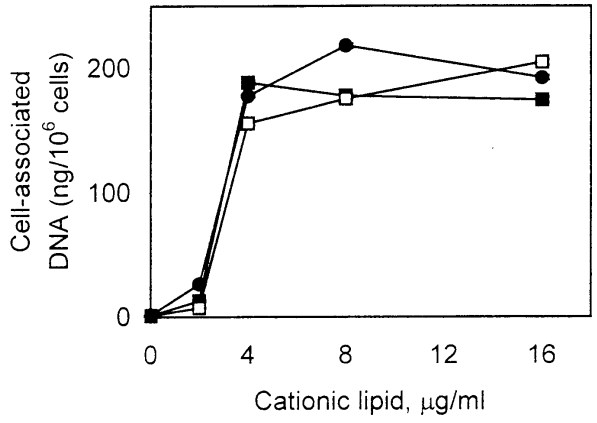

Figure 1 Uptake of CL–DNA complexes by wild-type, HS-deficient and PG-deficient CHO cells

Wild-type (■), HS-deficient (□) and PG-deficient (●) CHO cells were seeded at 10^5 cells per well for 24 h and then incubated with a mixture of CL and 1 μg/ml of [^{32}P]DNA plasmid at ratios from 1:1 to 16:1 (w/w), in F-12K medium without serum. After incubation for 4 h at 37 °C, cells were detached by treatment with trypsin, washed extensively with PBS, then lysed in cell lysis buffer. The amount of cell-associated [^{32}P]DNA was measured by scintillation counting. Results are means ± S.E.M. ($n = 6$); the error bars were smaller than the symbols.

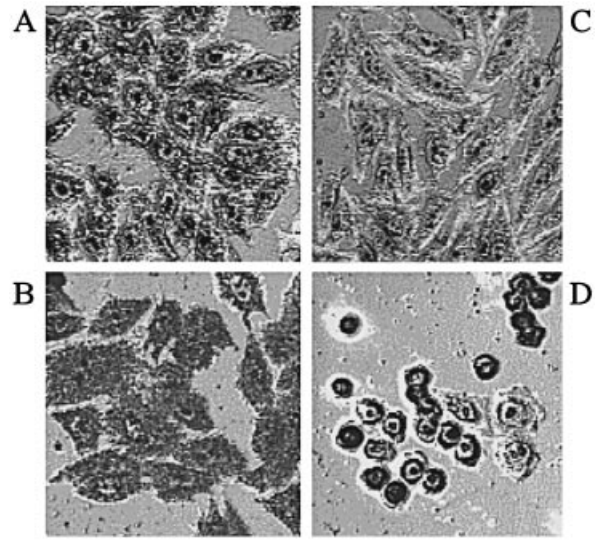

Figure 2 Increased susceptibility to CL cytotoxicity in PG-deficient cells

Subconfluent wild-type (**A**, **B**) and PG-deficient (**C**, **D**) CHO cell cultures were incubated with 1.0 μg/ml DNA plasmid either without CL (**A**, **C**) or with 16 μg/ml CL (**B**, **D**). After incubation for 4 h at 37 °C, cells were detached by treatment with trypsin and transferred to four-well coverslips (approx. 5×10^4 cells per well); this was followed by a growth period of 48 h. After rinsing and fixation, the cells were studied by confocal microscopy. The intensity in each pixel is the average of five consecutive scans.

At 4 μg/ml CL, the three cell lines exhibited similar DNA uptake levels, which is in accordance with the results in Table 1. A further increase in the amount of CL (8 and 16 μg/ml) had minor effects on DNA uptake, including in PG-deficient cells, which exhibited a marked decrease in reporter gene expression at these concentrations of CL. It should be pointed out that cells were incubated with CL–DNA complexes for 4 h in the DNA uptake experiments, whereas cells were grown for a further 48 h in the reporter gene expression assay.

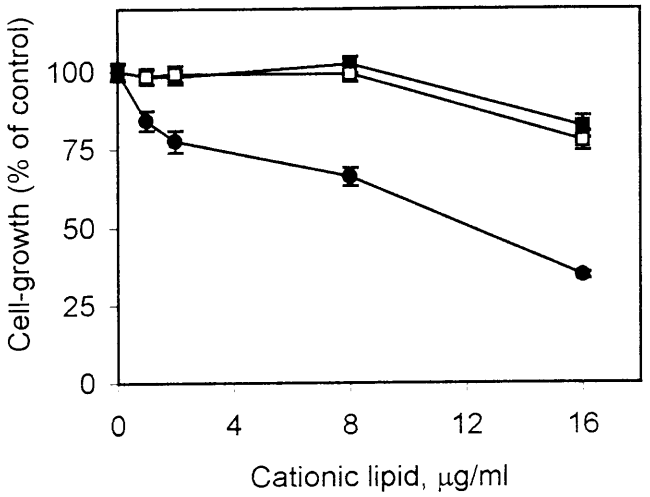

Figure 3 Effects of CL on cell-growth in wild-type, HS-deficient and PG-deficient CHO cells

Wild-type (■), HS-deficient (□) and PG-deficient (●) CHO cells were seeded at 3×10^3 cells per well for 4 h, followed by a starvation period in serum-free medium for 24 h. Cells were then incubated for 4 h at 37 °C in F-12K medium with no additions (control) or with 1, 2, 8 or 16 μg/ml CL. Medium was then aspirated, followed by another incubation period of 96 h in growth medium. Cell growth was measured by the Crystal Violet method and is expressed as a percentage of control. The control values (attenuances) were 3.2, 3.3 and 3.3 for wild-type, HS-deficient and PG-deficient cells respectively. Results are means ± S.E.M. ($n = 6$); in some cases the error bars were smaller than the symbols.

PG-deficient cells exhibit increased sensitivity to CL cytotoxicity

The foregoing results indicate that PG-deficient cells have an intact uptake of CL–DNA complexes over a wide range of CL concentrations, whereas the expression of the reporter gene plasmid is substantially decreased at the higher CL concentrations. To study the mechanism of these effects, wild-type and PG-deficient CHO cells were incubated with rhodamine-labelled DNA plasmid for 4 h, in either the absence or the presence of CL, followed by a growth period of 48 h. Cells were then viewed by confocal microscopy. In the absence of CL there was no internalization of DNA plasmid in either of the cell types, whereas in the presence of 16 μg/ml CL a substantial amount of rhodamine-labelled DNA was present in the intracellular compartment, as expected from the results in Figure 1. The amount of internalized DNA plasmid seemed to be equal in both cell types (results not shown). However, PG-deficient cells incubated with rhodamine-labelled DNA and 16 μg/ml CL displayed a significantly altered shape and size compared with wild-type cells. This prompted us to analyse the appearance of reflected light images of the respective cell lines that had been subjected to the same treatments as those described above (Figure 2). The presence of 16 μg/ml CL seemed to have no effect on the shape and size of wild-type cells (Figure 2B), whereas a substantial fraction of PG-deficient cells were dead, exhibiting a pyknotic and dense appearance (Figure 2D).

We then performed a cell-proliferation assay designed to mimic the conditions of the experiments described in Table 1 and Figure 2. Cells were incubated for 4 h in serum-free medium supplemented with various quantities of CL; the medium was then changed to growth medium. Cell growth was determined after a further 96 h. As shown in Figure 3, the proliferation of both wild-type and HS-deficient cells was unaffected by up to 8 μg/ml CL, whereas PG-deficient cells were growth-inhibited by

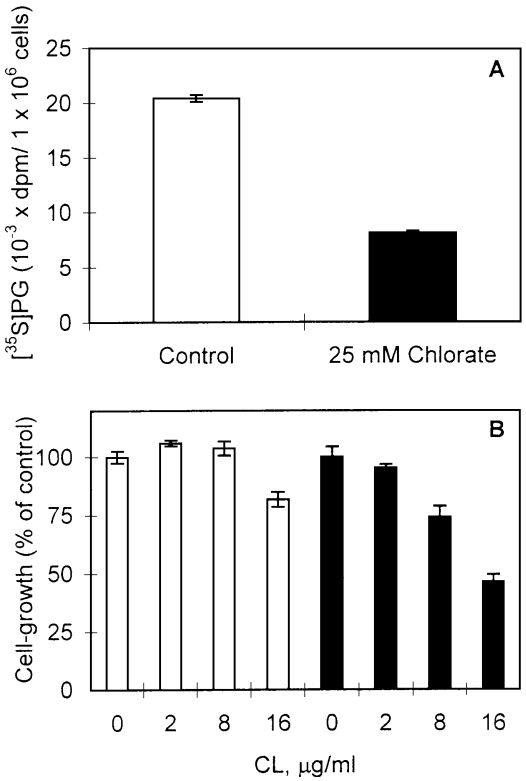

Figure 4 Increased growth inhibition by CLs in chlorate-treated wild-type cells

(**A**) Wild-type CHO cells were seeded in six-well plates at 2×10^5 cells per well and grown to subconfluence (approx. 8×10^5 cells per well). Cells were then preincubated for 1 h in sulphate-deficient $MgCl_2$ medium, followed by another incubation for a further 24 h either in $MgCl_2$ medium (control) or in F12 medium containing 25 mM chlorate. The medium was supplemented with 50 μCi/ml [^{35}S]sulphate. [^{35}S]PGs were then isolated from a detergent extract, as described in the Experimental section, and quantified by scintillation counting. (**B**) Wild-type CHO cells were seeded at 3×10^3 cells per well for 4 h, then starved for 24 h either in F12-K medium (open bars) or in F12 medium supplemented with 25 mM chlorate (filled bars). Cells were then incubated for 4 h at 37 °C in the respective medium with no additions or with 2, 8 or 16 μg/ml CL. Medium was then aspirated, followed by incubation for a further 96 h in growth medium. Cell growth was determined by the Crystal Violet method. Results are means $\pm$ S.E.M. ($n = 6$).

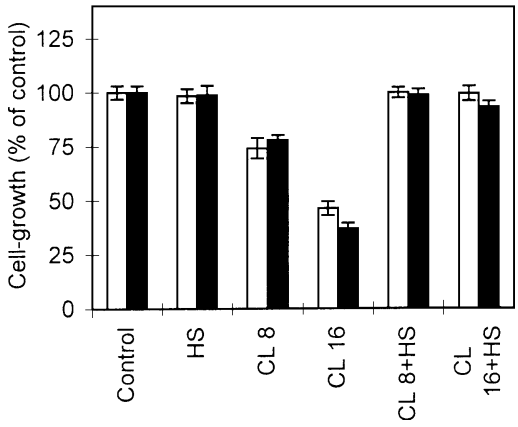

Figure 5 Neutralization of CL cytotoxicity by exogenous HS

PG-deficient (filled bars) and wild-type (open bars) CHO cells were seeded at 3×10^3 cells per well for 4 h and then starved for 24 h either in F-12K medium (PG-deficient) or F12 medium supplemented with 25 mM chlorate (wild-type). Cells were then incubated for 4 h in the respective medium supplemented with the indicated substances. Medium was aspirated; this was followed by incubation for a further 96 h in growth medium. Cell growth was measured by the Crystal Violet method and is expressed as a percentage of control. The concentrations of CL are presented as μg/ml; the concentration of HS was 50 μg/ml. The control values (attenuances) were 3.4 and 3.3 for PG-deficient and chlorate-treated wild-type cells respectively. Results are means $\pm$ S.E.M. ($n = 6$).

approx. 25% and 35% by 2 and 8 μg/ml CL respectively. At 16 μg/ml CL, wild-type, HS-deficient and PG-deficient cells were growth-inhibited by approx. 18%, 22% and 65% respectively.

The effect of CL on the growth of chlorate-treated wild-type cells was then investigated. Chlorate is an inhibitor of sulphate incorporation into glycosaminoglycan chains, resulting in undersulphated, i.e. less polyanionic, PG [32]. In wild-type CHO cells, [^{35}S]PG production was inhibited by approx. 60% on treatment with 25 mM chlorate (Figure 4A). As shown in Figure 4(B), the growth-inhibitory effect of CL was substantially greater in cells pretreated with 25 mM chlorate than in untreated cells, suggesting that the removal of polysulphated glycosaminoglycans resulted in increased CL cytotoxicity. It should be emphasized that cell-growth was not itself affected by 25 mM chlorate.

Reversal of CL cytotoxicity by exogenous glycosaminoglycans

Taken together, the foregoing results suggest that complexes with high ratios of CL to DNA, at which wild-type and HS-deficient cells exhibit maximal reporter gene expression levels, are toxic to PG-deficient cells, leading to a substantial decrease in cell growth and reporter gene expression. One possible explanation is that the polyanionic glycosaminoglycans sequester CLs, thereby counteracting the cytotoxic effects exerted by CLs. In another series of experiments the effects of exogenous HS on CL cytotoxicity in PG-deficient cells and chlorate-treated wild-type cells were studied (Figure 5). HS abrogated the growth-inhibitory effect of CLs in both chlorate-treated and PG-deficient cells. This effect was not specific for HS because both CS and DS were also able to neutralize completely the growth-inhibitory activity of CLs (results not shown), although at a higher concentration (100 and 75 μg/ml for CS and DS respectively). The reversal of CL cytotoxicity by sulphated glycosaminoglycans in chlorate-treated cells strengthens the conclusion that the effect of treatment with chlorate was related to a decrease in glycosaminoglycan sulphation.

DISCUSSION

It is has been debated whether polyanionic components, such as PGs, that are present in the extracellular compartment and at cell surfaces act as barriers and/or as 'receptors' in polycation-mediated gene transfer. The objective of this study was to evaluate the mechanism of the involvement of PGs in CL-mediated gene transfer. We find that PG-deficient cells are as efficient as wild-type cells at internalizing CL–DNA complexes, suggesting that cell-surface PGs are not critical for the internalization process. At low ratios of CL to DNA, PG-deficient cells exhibit even higher levels of CL–DNA complex uptake (2-fold and 3.5-fold) and reporter gene expression (8-fold and 123-fold) than wild-type and HS-deficient cells respectively. In contrast, at a high ratio of CL to DNA, reporter gene expression was substantially decreased in PG-deficient cells (approx. 13-fold higher expression in wild-type than in PG-deficient cells), whereas the other cell lines exhibited maximum expression levels, suggesting that PGs are required for optimal CL-mediated gene transfer *in vitro*. In a previous study [25] it was reported that polycation–DNA complexes enter cells via binding

to HSPG. Reporter gene expression was substantially decreased in PG-deficient cells compared with wild-type CHO cells, with either polylysine or CLs as gene transfer vehicles. In these experiments an optimal amount of polylysine and CL respectively was used. Moreover, experiments were not conducted to investigate the mechanism of the lower CL-mediated reporter gene expression levels in PG-deficient cells. The role of PG in CL-mediated gene transfer both *in vitro* and *in vivo* has been investigated recently [26]. CL–DNA complexes did not transfect Raji cells deficient in PGs but did efficiently transfect Raji cells stably expressing the HSPG syndecan-1. However, CL–DNA complex uptake by wild-type and syndecan-expressing Raji cells was not investigated. Furthermore, mice treated with either fucoidan, a sulphated polysaccharide not found in PGs, or an HS-degrading enzyme (heparinase I) before the intravenous injection of CL–DNA complexes exhibited decreased reporter gene expression levels. The authors concluded that PGs mediate CL-based gene delivery *in vivo*. In another study *in vivo* [33], optimal transfection efficiency was obtained at a low ratio of positive charges (supplied by the CL) to negative charges in DNA. A low net positive charge could be necessary for a facilitated diffusion of CL–DNA complexes within the extracellular compartment.

Spermine, which is a member of the polyamine family and a physiological counterion of DNA, constitutes the DNA-binding polycationic group of several types of CL, including DOSPA. Spermine binds to DS and HS chains with affinities that are respectively similar to or higher than that for DNA [34,35]. Moreover, we have shown recently that PGs are involved in polyamine uptake [36]. However, the uptake of CL–DNA complexes by PG-deficient cells was intact even at the highest CL concentration tested (the present study), suggesting that polyamines and polyamine-based CLs (lipopolyamines) are internalized by different mechanisms. The fact that polyamine transport-deficient CHO cells were efficiently transfected with DOSPA/DOPE (M. Belting, unpublished work) strengthens this conclusion.

By confocal microscopy and cell-growth experiments it was revealed that PG-deficient cells exhibit an increased sensitivity to the cytotoxic effects of CL compared with those of wild-type and HS-deficient cells. Accordingly, pretreatment of wild-type cells with chlorate resulted in a substantially greater anti-proliferative effect of CLs than that in untreated cells. The addition of exogenous glycosaminoglycans abrogated the cytotoxic effects of CLs in both PG-deficient cells and chlorate-treated wild-type cells. Our results indicate that, at relatively high CL concentrations, polyanionic PGs/glycosaminoglycans serve a protective role against CL cytotoxicity, thus allowing optimal transfection efficiency. Moreover, the fact that HS-deficient cells were transfected as efficiently as wild-type cells (which produce mainly HSPG) suggests that CS/DSPGs as well as HSPGs are able to protect cells against CL cytotoxicity.

At present more than 300 clinical gene therapy studies are in progress, of which approx. 20% use non-viral transfection systems [1]. A number of clinical studies, with CLs as a gene delivery vehicle, have already been published [37–39]. In our view it is crucial to establish the exact role of molecules that could potentially be involved in gene transfer *in vivo*, thus permitting the design of more efficient and specific gene delivery vehicles.

We thank S. Persson and A. Mårtensson for expert technical assistance, E. Eklund for helpful discussions, and Professor L.-Å. Fransson (Lund University, Lund, Sweden) for a critical reading of the manuscript. We acknowledge grants from the Swedish Medical Research Council (567, 11550) and from the Crafoord Foundation.

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Received 29 April 1999/7 June 1999; accepted 30 June 1999