Proteoglycan involvement in polyamine uptake

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We have evaluated the possible role of proteoglycans in the uptake of spermine by human lung fibroblasts. Exogenous glycosaminoglycans behaved as competitive inhibitors of spermine uptake, the most efficient being heparan sulphate ($K_i = 0.16 \pm 0.04\ \mu M$). Treatment of fibroblasts with either heparan sulphate lyase, *p*-nitrophenyl-*O*-β-D-xylopyranoside or chlorate reduced spermine uptake considerably, whereas chondroitin sulphate lyase had a limited effect. Inhibition of polyamine biosynthesis with α-difluoromethylornithine resulted in an increase of cell-associated heparan sulphate proteoglycans exhibiting higher affinity for spermine. The data indicate a specific role for heparan sulphate proteoglycans in the uptake of spermine by fibroblasts. Spermine uptake by pgsD-677, a mutant Chinese hamster ovary cell defective in heparan sulphate biosynthesis, was only moderately reduced (20%) compared with wild-type cells. Treatment of mutant cells with the above-mentioned xyloside resulted in a greater reduction of endogenous proteoglycan production as well as a higher inhibition of spermine uptake than in wild-type cells. Moreover, treatment with chondroitin sulphate lyase resulted in a selective inhibition of uptake in mutant cells, indicating a role for chondroitin/dermatan sulphate proteoglycans in the uptake of spermine by these cells. Fibroblasts, made growth-dependent on exogenous spermine by α-difluoromethylornithine treatment, were growth-inhibited by heparan sulphate or β-D-xyloside, which might have future therapeutical implications.

Key words: cell growth, α-difluoromethylornithine, heparan sulphate, spermine, xylosides.

INTRODUCTION

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous components of mammalian cells and they play an essential role for cell growth and differentiation [1–3]. Ornithine decarboxylase, the key enzyme in polyamine biosynthesis, is critical for cell transformation [4,5]. Specific inhibition of ornithine decarboxylase with α-difluoromethylornithine (DFMO) results in growth reduction and/or cell death in virtually every cell system that has been investigated [6,7]. In addition to endogenous synthesis, all cells possess an energy-dependent, carrier-mediated, saturable transport system for the uptake of polyamines from the extracellular environment. The activity of the transport system is greatly enhanced when intracellular polyamine levels are reduced, which may antagonize the effects of polyamine biosynthesis inhibitors when given as anti-tumour agents *in vivo* [8]. The fact that a mutant leukaemic cell line deficient in polyamine uptake was profoundly more susceptible to *in vivo* growth inhibition by DFMO as compared with the parental cell [9] supports this view. A polyamine transporter gene or protein has not been identified in mammalian cells. Hence, nothing is known about the features of a putative polyamine transporter at a structural level.

Proteoglycans (PGs) are a large family of macromolecules composed of glycosaminoglycan (GAG) chains covalently linked to protein [10–13]. PGs are fundamental components of cell surfaces, basement membranes and other extracellular matrices, and play an important role in the regulation of cellular proliferation, migration and differentiation [14]. For example, it has been shown that expression of heparan sulphate (HS)-carrying cell-surface PG is required for the binding of basic fibroblast growth factor to its signal-transducing receptor [15,16]. Heparin-binding epidermal growth factor-like growth factor is dependent on interactions with cell-surface HSPG to stimulate smooth-muscle-cell migration [17]. HS is also involved in the internalization of viruses [18], and it has been reported that gene complexes, consisting of polycations and DNA, enter cells via binding to membrane-associated HSPG [19,20]. Moreover, exogenously added GAGs, such as dermatan sulphate (DS) and highly-sulphated HS, inhibit growth of fibroblasts by an unknown mechanism [21].

Human fibroblasts produce PGs that predominantly contain two classes of GAG: DS, chondroitin sulphate (CS) or HS. The PG are found at the cell surface as well as in the extracellular matrix [11,12,22]. Cell-surface PGs are either intercalated in the lipid bilayer via membrane-spanning protein segments or lipid-anchored via glycosylphosphatidylinositol. The former type include the syndecan family. Fibroblasts synthesize one type of glycosylphosphatidylinositol-anchored HSPG, called glypican, that can be internalized by endocytosis and recycled back to the plasma membrane via the Golgi [22].

We have previously studied the interaction between spermine and DS or HS [23,24]. Spermine binds to specific regions in DS that possess anti-proliferative activity when released in oligosaccharide form (tetra- to decasaccharides). Growth-inhibitory HS chains can be subfractionated by affinity chromatography on spermine–agarose, yielding high-affinity material with increased anti-proliferative activity. However, there was no obvious correlation between sulphate content in HS and spermine-binding, or between sulphate content and anti-proliferative activity, suggesting sequence-specific interactions.

In the present study spermine uptake was studied in human embryonic lung (HEL) fibroblasts treated with either GAG lyases, chlorate, an inhibitor of sulphation, or *p*-nitrophenyl-*O*-β-D-xylopyranoside (Xyl-PheNO$_2$), which competitively inhibits endogenous PG synthesis [25,26]. The results demonstrate an

Abbreviations used: CHO, Chinese hamster ovary; CS, chondroitin sulphate; DFMO, α-difluoromethylornithine; DMEM/Ham's F-12, Dulbecco's minimal essential medium/Ham's nutrient mixture F-12; DS, dermatan sulphate; GAG, glycosaminoglycan; HEL, human embryonic lung; HS, heparan sulphate; MEM, minimum essential medium; NEM, *N*-ethylmaleimide; PG, proteoglycan; Xyl-PheNO$_2$, *p*-nitrophenyl-*O*-β-D-xylopyranoside.

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involvement of HSPG in spermine uptake. Surprisingly, uptake of spermine by pgsD-677, a mutant Chinese hamster ovary (CHO) cell defective in HS biosynthesis [27], was almost intact (80 % compared with wild-type cells) and appeared dependent on CS/DS PG.

EXPERIMENTAL

Materials

Spermine and Xyl-PheNO_2 were obtained from Sigma Chemical Co (Stockholm, Sweden). [^{14}C]Spermine (113 mCi/mmol) tetrahydrochloride, D-[6-^{3}H]glucosamine (26 Ci/mmol), and Na_2[^{35}S]SO_4 (1310 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. The HS preparation (HS6) was derived from beef lung as described elsewhere [28]. In brief, heparin byproducts were dissolved in 2.1 M NaCl and subjected to stepwise precipitation as cetylpyridinium complexes into subfractions with falling charge density. HS6 (M_r 20000) was obtained between 1.2 and 2.1 M NaCl. CS from bovine nasal cartilage was a gift from Professor D. Heinegård, Lund University, Lund, Sweden. Chondroitin ABC lyase (EC 4.2.2.4) and HS lyase (alias heparinase III or heparitinase, EC. 4.2.2.8) were from Seikagaku Corp., Tokyo, Japan. DFMO was a gift from Dr. L. Persson, Lund University, Lund, Sweden. Chlorate and Crystal Violet were from Merck, Kebo, Stockholm, Sweden. Minimum essential medium (MEM) and L-glutamine were obtained from ICN Biomedicals Inc., Chemicon, Malmö, Sweden, and donor calf serum, Costar cell culture plates and F-12 Nutrient Mixture were from Life Technologies AB, Täby, Sweden. Dulbecco's MEM/Ham's nutrient mixture F-12 (DMEM/Ham's F-12) was from Nordcell, Bromma, Sweden. Ready Safe™ scintillation cocktail and scintillation vials were products of Beckman AB, Bromma, Sweden, and Sarstedt, Landskrona, Sweden, respectively. HiTrap *N*-hydroxysuccinimide-activated column and Superose 6 HR 10/30 were obtained from Pharmacia-LKB Biotechnology, Uppsala, Sweden. All other chemicals were of analytical grade.

Cell culture

HEL cells were established as described [29] and routinely cultured in MEM supplemented with 10 % (v/v) new-born calf serum/2 mM L-glutamine/100 units/ml penicillin/100 μg/ml streptomycin (growth medium). Confluent cell cultures used for the experiments were between passages 12 and 18. CHO cells (CHO K1) and pgsD-677 (HS-deficient) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured in F-12K Nutrient Mixture with the same supplements as above. Cells were regularly checked for mycoplasma using GEN-PROBE Rapid detection system (Skafte and Claesson, Mölndal, Sweden).

Proliferation assay

The procedure has been described in detail elsewhere [30]. In brief, HEL cells were seeded at 5×10^3 cells/well in 96-well microplates (Greiner, In Vitro, Stockholm, Sweden) and allowed to adhere for 4 h in growth medium. Cells were starved in MEM for 24 h, and then incubated in growth medium with or without substances dissolved in the medium. Cell number was determined at days 4 and 5 by the Crystal Violet method [31].

Spermine-uptake experiments

For standard uptake assays, confluent cell cultures were harvested by a 1–2 min treatment with bovine pancreatic trypsin (0.5 mg/ml) and plated in 24-well culture plates at 4×10^4 cells/well in standard growth medium. Cells were grown for 3 days until subconfluency (approx. 1.5×10^5 cells/well) with or without test substances as indicated in appropriate Figure legends, growth medium was aspirated and cells were rinsed twice with 0.5 ml of MEM. Then 0.5 ml of MEM without serum containing various concentrations of spermine and [^{14}C]spermine (specific activity, 31 Ci/mol) were added. Following a 1 h incubation (unless stated otherwise) at 4 °C or at 37 °C under a 5 % CO_2 atmosphere, the incubation medium was removed, and cells were washed three times with 0.5 ml of ice-cold MEM containing 1 mM unlabelled spermine and then once with 0.5 ml of MEM. Cells were lysed with 0.5 ml of 0.5 M NaOH for 1 h at 37 °C, and a 0.4 ml aliquot of the homogenate was neutralized with 0.4 ml of 0.5 M HCl, and analysed for radioactivity by scintillation counting using an LKB Wallac Rackbeta Counter. The cell-associated spermine level obtained at 37 °C minus that obtained at 4 °C was defined as the temperature-dependent spermine uptake and was expressed as pmol/h per 10^6 cells. Cells were counted in a Bürker chamber. Kinetic parameters of uptake were determined by adding increasing concentrations of spermine and [^{14}C]spermine to cell cultures. K_m and V_{max} values were calculated from data presented as Lineweaver–Burke plots. Competition for uptake by HS was determined by varying the concentration of spermine (0.1, 0.2, 1, 5 and 10 μM) at a fixed concentration of HS. This experiment was repeated with different concentrations of HS (0.5, 2.5 and 5 μM). Plots of $1/V$ versus 1/[spermine] were then constructed, one for each concentration of HS, and K_i was determined using a Dixon plot [32]. Since HS is not a structural analogue of the substrate but assumed to be structurally similar to a putative transporter, the effect of HS did not fit into a classical definition of a competitive inhibitor. However, kinetically it behaved as a competitive inhibitor of spermine uptake.

Treatment with GAG lyases

Subconfluent monolayers of cells in 24-well plates (approx. 1.5×10^5 cells/well) were washed twice with 0.5 ml of MEM/20 mM Hepes, pH 7.4, 0.5 % BSA (digestion buffer) and incubated for 1 h at 37 °C with 0.5 ml of the digestion buffer containing 0.6 m-units of HS lyase or 25 m-units of chondroitin ABC lyase. Another aliquot of HS lyase or ABC lyase was then added to bring the concentration to 4.8 m-units/ml and 200 m-units/ml, respectively, and the incubation was allowed to proceed for another 2 h. Following the removal of cell-associated GAGs, cells were rinsed twice with 0.5 ml of the digestion buffer and spermine uptake was determined according to the usual protocol. The effect of enzymic treatment was monitored by the following procedure: cells were labelled with [^{35}S]sulphate (50 μCi/ml) in MEM for 24 h and then washed extensively with PBS (0.137 M NaCl/3 mM KCl/8 mM Na_2HPO_4/2 mM KH_2PO_4, pH 7.5). Enzymic treatments were performed as described above and aliquots of the digestion buffer were analysed for radioactivity. After removal of the digestion buffer and rinsing with PBS, the cell layer was dissolved in 0.15 M NaCl/10 mM EDTA/2 % (v/v) Triton X-100/10 mM KH_2PO_4, pH 7.5, containing 10 mM *N*-ethylmaleimide (NEM) and 1 mM di-isopropyl fluorophosphate for 10 min at 4 °C. The amount of residual [^{35}S]PG in cell extracts was estimated by Alcian Blue precipitation [33]. The amounts of radioactivity in digestion medium and cell extracts, respectively, were compared with untreated controls.

Treatment with Xyl-PheNO_2

Cells were seeded at 4×10^4 cells/well in 24-well plates and grown for 3 days with or without 5 mM DFMO. Then medium was

changed to growth medium with or without 5 mM DFMO supplemented with Xyl-PheNO_2 at concentrations as indicated, and cells were grown for an additional period of 24 h. The cell layers were washed three times with 0.5 ml of MEM before spermine-uptake assay was performed as described above. The effect of Xyl-PheNO_2 treatment on PG synthesis was determined by adding [^{35}S]sulphate (50 μCi/ml) during the incubation with Xyl-PheNO_2. The medium was removed and cells were rinsed with PBS followed by extraction with 0.15 M NaCl/10 mM EDTA/2 % (v/v) Triton X-100/10 mM KH_2PO_4, pH 7.5 containing 10 mM NEM and 1 mM di-isopropyl fluorophosphate for 10 min at 4 °C. The amount of [^{35}S]PG in cell extracts was estimated by Alcian Blue precipitation.

Chlorate treatment

Fibroblasts were seeded at 4×10^4 cells/well in 24-well plates and grown for 3 days to subconfluency. Medium was changed to NaCl-free DMEM/Ham's F-12 supplemented with 25 mM $NaClO_3$ and appropriate amounts of NaCl to obtain physiological ionic strength. Following an additional incubation period of 24 h, cells were rinsed twice with 0.5 ml of MEM and spermine uptake was measured for 1 h as described above. In order to determine the effect of chlorate treatment on the amount of PG, subconfluent cell cultures in 6-well plates (approx. 6×10^5 cells/well) were labelled with [^{35}S]sulphate (50 μCi/ml) in F-12 medium with or without 25 mM $NaClO_3$ for 24 h. The medium was aspirated and cells were extensively washed with PBS followed by extraction with 0.15 M NaCl/10 mM EDTA/2 % (v/v) Triton X-100/10 mM KH_2PO_4, pH 7.5 containing 10 mM NEM and 1 mM di-isopropyl fluorophosphate for 10 min at 4 °C. The amount of [^{35}S]PG in cell extracts was estimated by Alcian Blue precipitation. In another set of experiments, cells were labelled as above in F-12 medium followed by a chase for 24 h in F-12 medium with or without 25 mM $NaClO_3$ before Alcian Blue precipitation of cell extracts.

Isolation of radiolabelled PGs

Confluent HEL cells were trypsinized and seeded in 25 cm^2 flasks. Cells were allowed to plate for a period of 4 h in growth medium. Culture medium was changed to 4 ml of fresh growth medium containing [^{35}S]sulphate (50 μCi/ml) or [^{3}H]glucosamine (20 μCi/ml) followed by a growth period of 3 days to subconfluency with or without 5 mM DFMO. Medium was collected followed by two washings with 4 ml of ice-cold PBS. Cells were extracted with 2.5 ml of 0.15 M NaCl/10 mM EDTA/2 % (v/v) Triton X-100/10 mM KH_2PO_4, pH 7.5 containing 10 mM NEM and 1 mM di-isopropyl fluorophosphate for 10 min at 4 °C. The residual extracellular matrix was solubilized in 2.5 ml of 4 M guanidinium chloride/50 mM NaOAc, pH 5.8, containing 0.2 % (v/v) Triton X-100 and 10 mM NEM, at 4 °C overnight. PGs were isolated by Alcian Blue precipitation.

Nitrous acid scission of HSPG

Low-pH nitrous acid was prepared as described [34] and added to samples isolated by Alcian Blue precipitation. The reaction was allowed to proceed for 10 min at room temperature and then terminated by neutralization with 2 M Na_2CO_3. The digested material was lyophilized and desalted on a fast-desalting Sephadex G-25 10/10 column (Pharmacia-LKB) in water. Pooled fractions were lyophilized and prepared for affinity chromatography as described below.

Affinity chromatography on spermine–agarose

Isolated [^{35}S]sulphate- or [^{3}H]glucosamine-labelled PG derived from the matrix and a detergent (Triton X-100) lysate of the cells were analysed by affinity chromatography on a 1 ml HiTrap column coupled with spermine according to the description of the manufacturer. Samples were dissolved in 0.5 ml of 7 M urea/10 mM Tris/0.1 % Triton X-100, pH 8.0 (equilibration buffer) and applied to the column, which was eluted with a NaCl gradient going from 0 (fraction 10) to 1.5 M (fraction 120) in the same buffer at a rate of 0.5 ml/min. The effluent was collected in 0.5 ml fractions and analysed by scintillation counting.

Analysis of data

All data are presented as means ± S.E.M. Differences from the mean were tested for significance by means of Student's t test.

RESULTS

Competitive inhibition of spermine uptake by HS

Polyamine transport kinetics have not been characterized in HEL cells before. Uptake of radiolabelled spermine by HEL cells

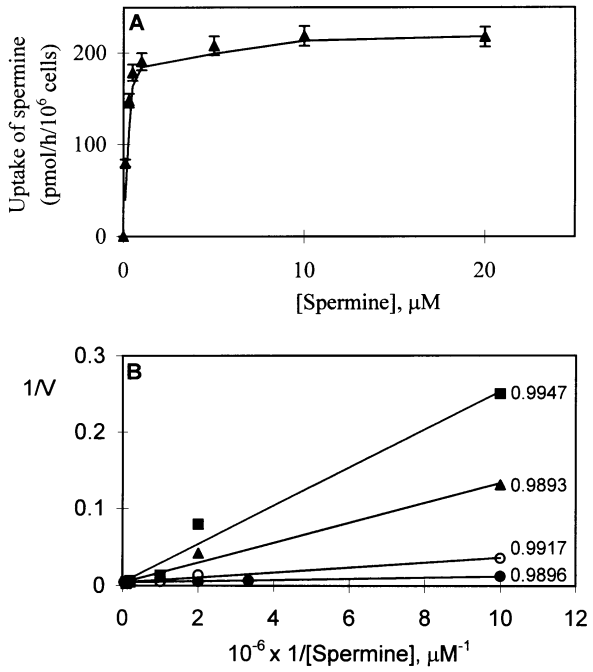

Figure 1 Effect of HS on spermine uptake

(**A**) Temperature-dependent [^{14}C]spermine uptake by HEL cells was determined in subconfluent 24-well plates (approx. 1.5×10^5 cells/well). Cells were incubated for 1 h at 37 °C at [^{14}C]spermine concentrations ranging from 0.1 to 20 μM (specific activity, 31 Ci/mol). Data have been corrected for spermine uptake at 4 °C. Results are the means ± S.E.M. of determinations from three separate experiments. (**B**) The rate of temperature-dependent [^{14}C]spermine uptake was determined in HEL cell cultures with increasing concentrations of spermine (0.1, 0.2, 1, 5 and 10 μM) in the presence of 0 (●), 0.5 (○), 2.5 (▲) or 5 (■) μM HS. Data are the means of triplicate determinations from a representative experiment and are plotted as $1/V$ versus $10^{-6} \times 1/$[spermine]. Figures on the right show correlation coefficients (ρ) for the respective regression lines.

Table 1 Effects of HS lyase, chondroitin ABC lyase or chlorate on spermine uptake

Uptake of [^{14}C]spermine (5 μM) was measured in subconfluent HEL cell cultures after no treatment (control) or after treatment with HS lyase, chondroitin ABC lyase or chlorate, respectively, as described in the Experimental section. Data are the means ± S.E.M. ($n = 6$).

Treatment	Spermine uptake (pmol/h per 10^6 cells)
None (control)	198 ± 0.9
HS lyase	80 ± 1.3
ABC lyase	175 ± 1.1
Chlorate	119 ± 2.0

was linear with time up to 200 min (results not shown); usually uptake experiments were carried out for 60 min. As shown in Figure 1(A), spermine uptake obeys simple Michaelis–Menten kinetics up to 20 μM with K_m and V_{max} values of 0.18 ± 0.02 μM and 228 ± 3.5 pmol/h per 10^6 cells, respectively. The ability of highly sulphated HS, exhibiting strong binding to spermine–agarose [24], to inhibit spermine uptake by fibroblasts was evaluated (Figure 1B). HS was a competitive inhibitor of uptake with a K_i value of 0.16 ± 0.04 μM, which is in the same range as the K_m of spermine uptake. DS, CS and low-sulphated HS were significantly weaker inhibitors of spermine uptake (results not shown).

Treatment of HEL cells with HS lyase or chlorate reduces spermine uptake

To study the role of PGs in polyamine uptake, cells were treated with either HS lyase, chondroitin ABC lyase or chlorate prior to spermine-uptake experiments. Treatment with HS lyase resulted in an approx. 61 % reduction of spermine uptake, whereas chondroitin ABC lyase treatment only gave a 10 % reduction of spermine uptake. Upon chlorate treatment, spermine uptake was inhibited by approx. 40 % (Table 1). Under the conditions used, chlorate treatment inhibited sulphate incorporation into newly synthesized PGs by approx. 85 %. However, a residual pool of cell-associated PGs, constituting approx. 35 % of the total cell-surface PGs, was unaffected by the presence of chlorate. This was determined by measuring the amount of [^{35}S]PG in chlorate-treated cells pre-labelled with [^{35}S]sulphate. This material was completely degraded by HS lyase, suggesting that it consisted of HSPG (results not shown).

Inhibition of spermine uptake by Xyl-PheNO$_2$

Xylosides at high concentrations compete with the xylose-substituted core protein as substrate for galactosyl transferase I, resulting in an inhibition of endogenous PG synthesis [25,26]. In HEL cells, 1 mM Xyl-PheNO$_2$ inhibited the synthesis of cell-associated PG by approx. 50 %, which is in agreement with a previous study on human embryonic skin fibroblasts [35]. Although Xyl-PheNO$_2$ is known to prime exclusively CS/DS chains, it inhibits the endogenous production of both CS/DS and HSPG [35]. Treatment of HEL cells with Xyl-PheNO$_2$ resulted in a dose-dependent reduction of spermine uptake with a maximal effect of approx. 50 % at 1 mM Xyl-PheNO$_2$ (Figure 2, open circles). In cells pre-treated with DFMO, spermine uptake was increased about nine-fold, and addition of 1 mM Xyl-PheNO$_2$ to DFMO-treated cells reduced spermine uptake by approx. 65 % (Figure 2, filled circles). The effect was not attributed to the presence of xyloside-primed GAG chains in the culture medium, since more than 90 % of this material was removed by extensive washing of the cells prior to spermine-uptake experiments (results not shown).

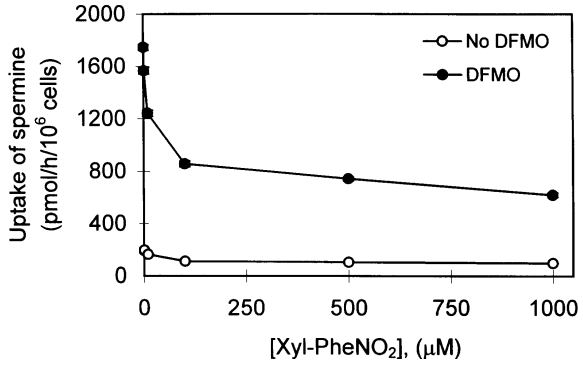

Figure 2 Effect of Xyl-PheNO$_2$ on the uptake of spermine

HEL cells were grown either in the presence (●) or absence (○) of 5 mM DFMO for 3 days. Then cells were incubated for another 24 h in the same media supplemented with various amounts (0, 1, 10, 100, 500 or 1000 μM) of Xyl-PheNO$_2$. After extensive washing with MEM, [^{14}C]spermine (5 μM, specific activity, 31 Ci/mol) uptake was measured for 1 h. Data are the means ± S.E.M. of three separate experiments. Error bars were smaller than the symbols.

Table 2 Effect of Xyl-PheNO$_2$ on the uptake of spermine by wild-type and mutant (HS-deficient) CHO cells

CHO K1 and pgsD-677 cells were grown for 3 days with or without 5 mM DFMO. Medium was changed to the same media with or without 1 mM Xyl-PheNO$_2$ and cells were incubated for another 24 h. Then [^{14}C]spermine (5 μM) uptake was measured for 20 min. Data are the means ± S.E.M. ($n = 6$).

Treatment	Cell type ...	Spermine uptake (pmol/h per 10^6 cells)	
		Wild type	HS deficient
None (control)		397 ± 34	311 ± 39
Xyl-PheNO$_2$		302 ± 29	153 ± 22
DFMO		1659 ± 35	1232 ± 36
DFMO + Xyl-PheNO$_2$		1178 ± 35	481 ± 34

Spermine uptake by mutant cells defective in HS synthesis

The results with HEL cells indicated a specific role for HSPG in spermine uptake. To demonstrate that PGs play a role in spermine transport by other cell lines and to evaluate the specificity of HSPG, uptake was studied in wild-type CHO K1 cells and in mutant pgsD-677 cells defective in HS biosynthesis. Spermine uptake was linear with time up to 30 min in CHO cells (results not shown). Thus, uptake experiments were carried out for 20 min. As shown in Table 2, spermine uptake in mutant cells (311 pmol/h per 10^6 cells) was reduced by approx. 20 % compared with wild-type cells (397 pmol/h per 10^6 cells). In another series

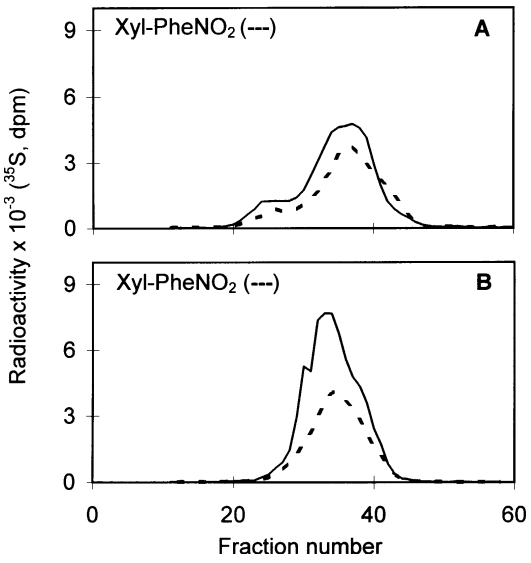

Figure 3 Effect of Xyl-PheNO$_2$ on PG production in wild-type and mutant CHO cells

Gel chromatography on Superose 6 of cell-associated [^{35}S]PG, isolated by detergent extraction followed by Alcian Blue precipitation, from CHO K1 (**A**) and pgsD-677 cells (**B**) after no treatment (—) or treatment with 1 mM Xyl-PheNO$_2$ (---) for 24 h in the presence of [^{35}S]sulphate (50 μCi/ml). The column was run in 4 M guanidinium chloride/50 mM NaOAc, pH 5.8, 0.2% Triton X-100, at a flow rate of 0.4 ml/min. Fractions (1 min) were collected and analysed for radioactivity by scintillation counting.

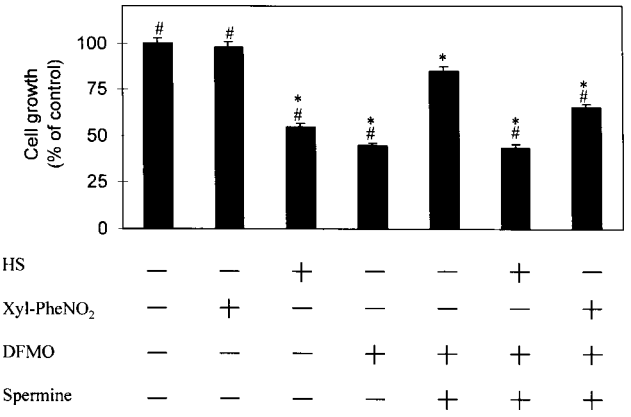

Figure 4 Effect of HS and Xyl-PheNO$_2$ on the reversal of DFMO-induced growth-inhibition by exogenous spermine in lung fibroblasts

Cells were seeded (5×10^3 cells/well) in 96-well plates and grown in standard growth medium with no addition (control) or with the additions as indicated in the Figure. Concentrations used were 5 mM DFMO, 0.1 μM spermine, 5 μM HS and 1 mM Xyl-PheNO$_2$, respectively. At day 4 cell number was estimated by measuring the amount of Crystal Violet dye adsorbed. Data are the means $\pm$ S.E.M of three different experiments. *, Significant difference from control ($P < 0.001$); #, significant difference from DFMO + spermine alone ($P < 0.001$).

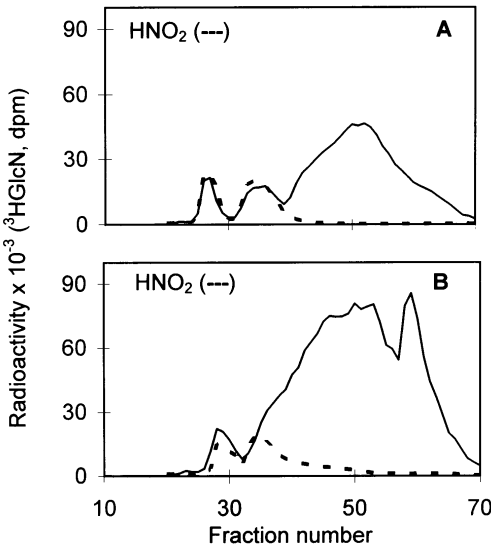

Figure 5 Affinity chromatography on spermine–agarose of PGs extracted from cells treated with DFMO

HEL cells were seeded in 25 cm^2 culture flasks and grown for 3 days to subconfluency in standard growth medium, supplemented with 20 μCi/ml [^{3}H]glucosamine, either in the absence (**A**) or in the presence of 5 mM DFMO (**B**). PGs isolated from a detergent lysate of the cells by Alcian Blue precipitation were chromatographed on spermine–agarose before (—) or after (---) scission with low-pH nitrous acid, as described in the Experimental section. The results presented are representative of two independent experiments.

of experiments the effect of Xyl-PheNO$_2$ on spermine uptake in CHO K1 and pgsD-677 cells was investigated. Xyloside treatment inhibited spermine uptake by approx. 21% and 52% in wild-type and mutant cells, respectively (Table 2). Treatment with DFMO upregulated uptake about four-fold in both cell types. In this case, xyloside treatment inhibited spermine uptake by approx. 29% and 61% in wild-type and mutant cells, respectively.

Accordingly, xyloside treatment caused a significantly greater reduction of endogenous, cell-associated PG production in mutant cells (approx. 55%) compared with wild-type cells (approx. 23%), as shown in Figure 3. [^{35}S]PG isolated from wild-type cells was separated into two components on Superose 6 (Figure 3A), whereas mutant cells contained exclusively the smaller component (Figure 3B), although the total amount of PG was approx. 30% higher in HS-deficient cells. As expected, treatment of mutant cells with HS lyase had no effect on spermine uptake, whereas treatment with ABC lyase inhibited spermine uptake by approx. 45% (results not shown).

HS and Xyl-PheNO$_2$ inhibit proliferation of fibroblasts made growth-dependent on exogenous spermine by DFMO treatment

As shown in Figure 2, polyamine-synthesis inhibition of HEL cells with DFMO resulted in a substantial (nine-fold) up-regulation of spermine transport. Addition of 1 mM Xyl-PheNO$_2$ inhibited spermine uptake by approx. 65%. The effects of HS and Xyl-PheNO$_2$ on growth of untreated HEL cells and cells made dependent on spermine uptake by DFMO treatment were then investigated (Figure 4). Treatment with xyloside alone did not affect growth, whereas HS was growth-inhibitory, as shown previously [21]. Polyamine-biosynthesis inhibition with DFMO resulted in reduced growth (45% of control). Growth of DFMO-treated cells was restored to 85% of control in the presence of 0.1 μM spermine. Addition of HS to DFMO-treated cells supplemented with 0.1 μM spermine reduced growth back to the level obtained with DFMO-treatment alone. Whereas 1 mM Xyl-

$PheNO_2$ had no effect on cells not treated with DFMO, almost half of the growth, dependent on exogenous spermine, was inhibited. This effect could either be due to the reduction of cell-associated PGs and/or to the presence of xyloside-primed GAG chains in the growth medium. In a control experiment, conditioned medium from cells grown for 4 days either in the absence or in the presence of 1 mM Xyl-$PheNO_2$ was re-isolated and used in the spermine-uptake studies. There was no significant difference in spermine uptake (results not shown).

PG production in HEL cells subjected to polyamine-synthesis inhibition

To study the spermine affinity of cell-surface PGs in control cells and cells treated with DFMO, [^{3}H]PG was isolated by Alcian Blue precipitation and then analysed by affinity chromatography on spermine–agarose. First, treatment with DFMO resulted in an altered distribution of PG between the extracellular and the cell-associated pools. The amount of PG deposited in the matrix of DFMO-treated cells was 60 % compared with control (results not shown), whereas the amount of cell-associated PGs increased to 180 % of control. Secondly, as shown in Figure 5 (solid lines), cell-associated PGs produced by cells grown in the presence of DFMO (Figure 5B) comprised variants with stronger binding to spermine than PGs from untreated cells (Figure 5A). The high-affinity component from DFMO-treated cells eluted at 0.8 M NaCl. The pools of cell-associated PGs from control cells and DFMO-treated cells, respectively, were cleaved with nitrous acid, which breaks HS at the glycosidic bond between N-sulphated glucosamine and uronic acids. The products were desalted and re-applied to the spermine–agarose column (Figure 5, dashed lines). The results showed that the major portion of the high-affinity material from control cells and DFMO-treated cells consisted of HSPG. Furthermore, DFMO treatment resulted in increased levels of HSPG with greater affinity for spermine. The same result was obtained with cells labelled with [^{35}S]sulphate (results not shown).

DISCUSSION

Although polyamine transport has been extensively studied with respect to substrate specificity, energy dependence and mono- and bivalent cation-dependence, no molecular information about the putative transporter is yet available. Attempts have been made to specifically inhibit polyamine uptake using polyamine analogues with high affinity for the polyamine carrier without being used as substrates [9,36]. An alternative strategy to inhibit polyamine transport would be to design compounds with structural resemblance to a putative transporter. This would require detailed information about the transporter.

We have provided evidence indicating a role for cell-surface PGs in the uptake of spermine by fibroblasts and CHO cells. Highly sulphated HS from beef lung behaved as a competitive inhibitor of spermine uptake with a K_i strikingly close to the K_m for spermine transport. The interaction between spermine and HS was investigated in a previous study [24], showing that spermine binds to HS with a K_d of 0.37 μM, also in the same range as K_m for transport. DS, CS and low-sulphated HS were significantly weaker inhibitors of polyamine uptake (this study), consistent with higher K_d values for their interaction with spermine [23,24]. Fibroblasts treated with HS lyase showed a marked reduction of spermine uptake, whereas a very limited effect was seen with chondroitin ABC lyase, which implicates cell-surface-associated HSPG in the uptake of spermine. However, CHO cells with a defect in HS biosynthesis had an efficient spermine uptake. In this case, uptake was dependent on the presence of CS/DS at the cell-surface. Several cell-surface PGs contain both HS and CS, notably members of the syndecan family and betaglycan [10,11,14]. It is thus possible that some of these PGs carry only CS/DS in the mutant cells. Although glypican is known to contain only HS, substitution with CS/DS must also be considered, as mutant and wild-type cells express similar levels of glypican mRNA (M. Belting and M. Jönsson, unpublished work).

Treatment with DFMO results in depletion of intracellular polyamine pools and inhibition of cell growth. Growth-inhibition of fibroblasts was almost completely reversed by spermine at concentrations as low as 0.1 μM due to up-regulation of spermine transport (see [1]). Interestingly, Xyl-$PheNO_2$ inhibited growth only when cells were dependent on the uptake of exogenously added spermine. This effect appeared to be due to reduction of cell-associated PGs.

We also find that inhibition of polyamine synthesis results in reduced levels of PG in the extracellular matrix, and an increase of cell-associated HSPG. The latter include a separate component with exceptionally high affinity for spermine. It is tempting to speculate that the up-regulation of spermine transport in DFMO-treated cells is directly related to the production of HSPG with stronger binding to spermine.

The role of cell-surface PGs could be two-fold. PGs may serve to attract and present polyamines to the actual transporter, or PGs may be an integral part of the transport complex. Future studies should be directed to an identification of the PG core protein, the nature of its membrane attachment and the possible involvement of recycling PG species. The present results also point to connections between intracellular polyamine levels and regulation of GAG biosynthesis.

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