Glycosaminoglycans are differentially involved in bacterial binding to healthy and cystic fibrosis lung cells

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Abstract

Background: Glycosaminoglycans (GAGs) are essential in many infections, including recurrent bacterial respiratory infections, the main cause of mortality in cystic fibrosis (CF) patients.

Methods: Using a cellular model of healthy and CF lung epithelium, a comparative transcriptomic study of GAG encoding genes was performed using qRT-PCR, and their differential involvement in the adhesion of bacterial pathogens analyzed by enzymatic degradation and binding competition experiments.

Results: Various alterations in gene expression in CF cells were found which affect GAG structures and seem to influence bacterial adherence to lung epithelium cells. Heparan sulfate appears to be the most important GAG species involved in bacterial binding.

Conclusions: Adherence to lung epithelial cells of some of the main pathogens involved in CF is dependent on GAGs, and the expression of these polysaccharides is altered in CF cells, suggesting it could play an essential role in the development of infectious pathology.

Abbreviations
CF, cystic fibrosis; CS, chondroitin sulfate; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; HS, heparan sulfate; PG, Proteoglycan.

1. Introduction

Almost all patients with cystic fibrosis (CF) develop chronic airway disease, whereby the airway tract becomes clogged by mucus, and re-current infections result. An inflammatory over-response is triggered by the persistent infections, involving excessive recruitment of neutrophils and the release of hydrolytic enzymes, leading to the destruction of the lungs [1,2].

Various types of chronic bacterial infections are implicated in such respiratory failure [3,4]. In chronic airway disease, the respiratory tractis colonized by a wide variety of microorganisms throughout life, *Staphylococcus aureus* and non typable *Haemophilus influenza* being the most predominant bacteria in children. Although in CF adults *S. aureus* remains the second most prevalent organism, over time patients become more susceptible to a range of Gram-negative bacteria, commonly *Pseudomonas aeruginosa*, which causes chronic infections in over 80% of CF patients. *Pseudomonas* can change to a mucoid phenotype by producing the polysaccharide alginate, which coats the bacterium and offers protection against host immune response. The appearance of the mucoid phenotype is associated with a more acute and persistent inflammatory response, and worse lung function [5]. Another typical pathogen in CF infections is *Burkholderia cepacia* which, despite its low incidence, causes high mortality due to its intrinsic resistance to multiple antibiotics [4,6].

The first critical step in the infectious processes is the pathogen's adhesion to host tissue. There are various molecules at the cell surface that could act as mediators in pathogen binding, notably proteoglycans (PGs), which are glycoconjugates containing a protein moiety covalently bound to polysaccharide chains. PGs are ubiquitous, but they are mainly located on the cell surface and at the extracellular matrix [7]. Their saccharidic portion is composed of long unbranched linear chains composed of repeating disaccharide units, called glycosaminoglycans (GAGs). There are various types of GAG, each differing in its chemical structure, and two of them, heparan sulfate (HS) and chondroitin sulfate (CS) are usually part of the cell surface PG complement. Both are composed of a residue of glucuronic acid (GlcA), but differ in that HS contains N-acetylglucosamine (GlcNAc), while CS has a residue of N-acetylgalactosamine [7].

CS and HS biosynthesis share a common initial phase, the formation of a tetrasaccharide linker composed of xylose, two galactoses and a GlcA (Fig.1). Subsequently, polymerization of the HS chain is carried out by two polymerases, which alternately introduce the residues GlcA and GlcNAc, while the synthesis of the CS chains is accomplished by a set of synthases and a polymerization factor. After polymerization, GAG chains may undergo a series of chemical reactions, mainly sulfations, that produce an enormous structural diversity (Fig.1) [8]. At the end of their biosynthesis, HS chains are structured into different domains, highly sulfated or poorly sulfated, separated by intermediate domains [9]. In the case of CS, different types of disaccharide units are generated, creating, generally, hybrid chains, although with no domain structure [8]. Cells express and modify the structure of GAGs depending on the tissue and developmental status, as well as physiological and pathological conditions [7,10].

GAGs are involved in a wide variety of biological processes, essentially acting as specific mediators of interactions between molecules. The result is a broad range of essential effects on cellular physiology [7,11]. Additionally, GAGs are also implicated in many pathological processes, including cancer and amiloid diseases [7,12–15] and are also involved in the development of numerous infectious pathologies, including those affecting the lungs [15–17].

The aim of this work was to determine whether there are changes in GAG chains in CF lung epithelial cells compared to healthy cells, and, if so, their influence on microbial adhesion. Since the chemical structure of GAGs is directly controlled by the levels of the genes encoding the enzymes involved in their biosynthesis, changes in their transcription were analyzed. The role that GAGs play in the adhesion of pathogenic bacteria commonly involved in CF was analyzed in healthy and in CF epithelial cells. Ultimately, the aim of this research was to increase our knowledge of the molecular basis of the infectious processes associated with the development of CF.

2. Material and methods

2.1. Cell Lines, Bacterial Strains and Culture Conditions

The bronchial epithelial cell lines used in this work were NuLi-1 (ATCC® CRL-4011) as a model of healthy epithelium, and CuFi-1 (ATCC® CRL-4013 ™), which carries the mutation p.Phe508del/p.Phe508del and can act as a CF airway disease model [18]. The two lines were grown in BEGM™ supplemented with SingleQuots™ Kit (Lonza Walkersville, Inc) at 37 °C in a 5% CO2 atmosphere.

The ATCC strains P. aeruginosa (ATCC 27853), H. influenzae (ATCC 10211), S. aureus (ATCC 29213) and B. cepacia (ATCC 25416) were obtained from the Colección Española de Cultivos Tipo (Valencia, Spain). Clinical isolates of P. aeruginosa (non- and mucoid phenotypes), H. influenzae, S. aureus and B. cepacia were provided by the

Hospital Universitario Central de Asturias (Oviedo, Spain). All bacteria were grown in Brain-Heart Infusion broth (Pronadisa) at 37 °C in a shaking incubator, except B. cepacia and H. influenzae which were grown in a 5% CO_2 atmosphere without shaking.

2.2. Fluorescein Labeling

Bacteria labeling with fluorescein isothiocyanate (FITC, Sigma Aldrich) was performed according to a previously described protocol [19].

2.3. Adherence Assays

Bacterial adhesion was tested in NuLi and CuFi monolayers in 24-well plates grown to 80% confluence. Cells were blocked with 10% fetal bovine serum in PBS for 2 h at 37 °C in a 5% CO_2 atmosphere. After further washing with PBS, a mixture of 100 µl of FITC-labeled bacteria and 400 µl of BEGM™ was added and incubated for 1 h in the same conditions. Unbound bacteria were removed with PBS washes. Epithelial cells were disaggregated with 1% SDS, and the fluorescence of the labeled bacteria attached was quantified in a Perkin Elmer LS55 fluorometer set at 488 nm (excitation) and 560 nm (emission).

Data were normalized using the adhesion values without any additive or treatment. Assays were performed at least in triplicate and the data are expressed as the mean ± SD.

2.4. Enzymatic Digestion of epithelial cell-surface GAGs

A 500 mU/ml mixture of heparinases I and III and 250 mU/ml of chondroitinase ABC (final concentrations) were used to digest HS and CS, respectively. Both treatments were performed in BEGM™ for 3 h at 37 °C in a 5% CO_2 atmosphere. The reactions were stopped with 2 washes with PBS, then adherence assays were performed as described in the previous paragraph.

2.5. Adherence Inhibition Assays

The effect of GAGs on adherence interference experiments was evaluated through the addition of either HS, or an equimolar mixture of CS-A, CS-B and CS-C (Merck), at concentrations ranging between 0.01 and 5 µg/ml, to the labeled bacteria before their addition to the monolayers.

2.6. RNA Isolation, cDNA Synthesis, and qRT-PCR Reactions

Isolation of the RNA from lung cells and cDNA synthesis were performed at least in triplicate using the RNeasy kit (Qiagen) and the High Capacity cDNA Transcription Kit (Applied Biosystems) respectively, according to the manufacturers' instructions.

At least 3 repetitions of qRT-PCR reactions and data analysis were carried out as previously described [20]. Glyceraldehyde 3-phosphate dehydrogenase was used as a control gene.

2.7. Statistical analysis

All analyses were performed using the Statistics program (Statsoft Inc). Mean values between two samples were compared using the Mann-Whitney U test, and between multiple samples using the Kruskal-Wallis test. $p < 0.05$ was accepted as significant. All data are presented as means ± standard error.

3. Results

3.1. Differential expression of genes involved in the biosynthesis of HS and CS

Despite the existence, in certain cases, of regulation at the level of translation or enzymatic catalysis, GAG structures are largely determined by the expression of specific isoforms of some of the biosynthetic enzymes involved, and this system is controlled by the cell type and its pathological and physiological state. Based on these considerations, a transcriptomic analysis was performed on NuLi and CuFi cells to determine possible structural differences in CS and HS chains.

The biosynthesis of these GAGs share a common initial phase: the formation of a tetrasaccharide linker. There are 9 genes involved, 7 which encode several glycosyltransferases, and another 2 which control chain formation through the phosphorylation and dephosphorylation of the first xylose residue (Fig.1). No significant changes between the two cell lines were identified in terms of the transcription levels of any of these genes (Fig. 2A).

Regarding CS chains, the results showed several transcriptional alterations in CuFi cells relative to NuLi cells. The two genes encoding N-acetylgalactosaminyltransferases, which is responsible for initiating the polymerization of the saccharidic chain, CSGALNACT1 and CSGALNACT2 (Fig. 1), displayed decreases in mRNA levels of around 80% and 50% respectively. Moreover, alterations in the mRNA levels of two genes encoding the sulfotransferases involved in the generation of the fine structure of the molecule, CHST12 and UST, were detected (Fig. 2B).

In contrast to CS, none of the genes involved in the polymerization of the HS chains showed any alterations in CuFi cells. Nevertheless, when evaluating the genes involved in the modification of the chains (Fig. 1), significant differences in the transcription levels of 3 of them were detected. Two 3-O-sulfotransferase encoding genes were affected: HS3ST2 decreased around 60%, while HS3ST3A1 showed a nearly fourfold overexpression. In addition, a reduction of around 85% in the levels of expression of the extracellular endosulfatase SULF1 was detected (Fig. 2C).

3.2. GAGs are involved in the adherence of pathogenic bacteria to lung cells

To determine the involvement of GAGs in bacterial adhesion, they were removed from CuFi and NuLi cell surfaces through digestion with bacterial lyases, and their effect on the binding of the different pathogens determined. The enzymes used for the digestions were chondroitinase ABC, and a mixture of heparinase I and III.

Analysis of the binding of H. influenzae, S. aureus and B. cepacia showed varying decreases in their adherence levels, suggesting that GAGs are involved in a general way in bacterial binding to lung epithelia cells. HS displayed a stronger effect than CS on the adherence of S. aureus strains, with similar values found in both cell lines (Fig. 3A). However, for H. influenzae and B. cepacia the observed effect was highly dependent on cell type, with significant differences found between the healthy and the CF lines (Fig. 3B and C). No significant differences were found between clinical isolates and ATCC strains, excepting for B. cepacia after treatment with heparinases, where inhibition values in the clinical isolate were lower, although the differences detected between cell lines remained (Fig. 3A, B and C).

The behavior of P. aeruginosa, interestingly, was very dependent not only on the cell type tested, but on the bacterial phenotype involved. The enzymatic reduction of the GAG levels on the cell surface did not reduce binding of non-mucoid P. aeruginosa to either cell line, and, in fact, a slight increase in adhesion was detected. Although some differences were observed between the clinical and the ATCC strains, none changed the

general pattern observed (Fig. 3D). In contrast, however, the adherence of the mucoid P. aeruginosa strain was reduced by the treatments, as for the other bacterial species analyzed. Again, statistically significant differences were detected between healthy and CF cells, with lyase digestion affecting the latter to a lesser extent (Fig. 3E).

In summary, the data show that cell surface GAGs are involved in the binding of pathogens, but the effect is different in NuLi and CuFi cells, with the exception of S. aureus. The results also indicate that, in the case of P. aeruginosa, the mucoid phenotype, responsible for CF-associated infections, uses GAGs as receptors, while the non-mucoid one does not.

3.3. Different GAGs are differentially involved in bacterial binding

The role of the different GAG species in bacterial adherence was studied by means of adherence experiments using commercial GAGs as competitors: HS and an equimolar mixture of CS-A, CS-B and CS-C.

Both GAGs influenced the binding of S. aureus, albeit with a different pattern (Fig. 4A and 5A). The addition of CS produced similar inhibitory effects in both cell lines, while HS caused a greater, though differentiated, reduction in bacterial adherence, being a more effective interference molecule in CuFi cells (Fig. 4A).

The presence of HS or CS at low concentrations dramatically diminished the binding of H. influenzae to NuLi in analogous values, their effect being greater on the adherence of the clinical isolate. On the other hand, the binding of both strains to CuFi cells was reduced by the two species of GAGs, to a similar, low, value (Fig. 4B and 5B).

None of the GAGs was able to compete for the adhesion of clinical isolate B. cepacia to NuLi (Fig. 4C), but they showed a slight effect on the binding of the ATCC strain (Fig. 5C). However, adherences of both strains to CuFi cells were reduced by both GAGs (Fig. 4C and 5C); specifically, the binding of clinical isolate strain HS showed a strong effect at low concentrations, while CS produced a more progressive inhibition whereby its effect increased according to the concentration, and reaching values comparable to HS at high concentrations (Fig. 4C).

In the case of P. aeruginosa, the results again varied depending on the phenotype and cell line tested. The presence of competing HS or CS molecules did not produce any detectable inhibition in the adherence of clinical isolate of P. aeruginosa non-mucoid phenotype to CuFi cells (Fig. 4D), whereas a small effect was detected for the adherence of the ATCC strain (Fig. 5D). In contrast, the binding of both strains to NuLi cells was inhibited by GAGs, especially by HS (Fig. 4D and 5D). Regarding the adherence of P. aeruginosa mucoid phenotype, CS displayed similar effects on adherence to both cell types. The presence of HS, on the other hand, had different effects in the two lines: a slight alteration to adhesion to NuLi, but a dramatic effect on binding to CuFi (Fig. 4E).

4. Discussion

NuLi and CuFi cells have been previously validated as suitable in vitro models for healthy and CF epithelium, respectively [18]. This comparative transcriptomic study between the two cell lines identified some differences in the expression of certain genes involved in the biosynthesis of the structure of their GAGs, suggesting that CS and HS chains become altered at specific locations in their sulfation pattern, and that CS chains were also affected at the polymerization level.

In CF cells and tissues, as well as in fluids such as sputum and bronchoalveolar lavage, some GAG alterations have been previously reported which involve a significant increase in their levels of sulfation [12,21,22]. Although the modifications observed at

the level of transcription in this study point to specific changes rather than to a generalized alteration of sulfation patterns, some of the differences described might be due to additional post-transcriptional mechanisms [23], or to the cell types chosen as the in vitro model [18,24]. As a result of the modifications in the structure of GAGs suggested by our transcriptional analysis, the affinity of these saccharide chains for multiple ligands might be affected, which could have diverse consequences on the regulation of many physiological processes, and might also involve variations in their interactions with bacterial pathogens if they were to be used as microbial receptors.

Strains from the ATCC and from clinical isolates of each bacterium, except P. aeruginosa mucoid, were used during this work with very similar results except for slight changes in non-mucoid P. aeruginosa and B. cepacia, and this did not vary greatly from the general pattern of binding observed in the two cell lines. These microorganisms adapt the expression of virulence factors and diverse adhesins according to the stage of infection or their degree of adaptation to epithelia [25,26]. The involvement of GAGs in the attachment of bacteria to healthy and CF cells was analyzed by removing them from cell surfaces, as well as by competition adherence assays, both of which evidenced a decrease in the adherence of all bacteria to both cell lines, except for the P. aeruginosa non-mucoid phenotype, where binding slightly increased and appeared to be independent of GAG involvement. Interestingly, both HS and CS were able to influence the attachment of mucoid P. aeruginosa, H. influenzae and B. cepacia in specific ways that were dependent on the cell line involved, suggesting that the structural differences between the GAGs of healthy and CF cells could be responsible for the effects on adhesion observed here. Interestingly, S. aureus presented a different model in which the influence of the different GAG species was very similar in both cell lines, suggesting that the adhesion of this bacterium is less dependent on specific sulfation patterns. The data clearly showed the importance of GAGs in bacterial adherence, although further experiments are needed to prove whether other types of cellular receptors also participate in the process. Cooperation between GAGs and diverse molecules, mainly proteins, has previously been described, indicating that they form a ternary complexes, which increases their affinity for ligands [27,28]. Overall, the results obtained indicate the participation of the two major species of cell surface GAGs, HS and CS, in the adherence of the majority of pathogenic microorganisms related to CF analyzed here, although the impact of HS generally seems to have more importance. Both GAGs have been described as mediators in the binding of multiple types of microorganism, including bacteria, viruses and parasites, to different epithelia, including cells of the respiratory tract [15-17].

Another interesting aspect of the results obtained in this work was the great influence, in the case of P. aeruginosa, that the phenotype of the strain involved had on the model of adhesion to the lung epithelial cells. The adherence of bacteria with a mucoid phenotype seems to be mediated, as for the other microorganisms analyzed, by binding to GAGs. However, the adherence of the non-mucoid phenotype was not affected by the competition or degradation of the GAG. What is more, enzymatic digestion resulted in an increase in adherence, which might be due to this resulting in the exposure of different receptors that have a stronger affinity for bacterial adhesins. P. aeruginosa exploits several mechanisms to attach to host cells, and it is able to bind differentially to diverse receptors depending on the side of the polarized epithelial cell involved [16].

More than 80% of adult patients with CF are infected with P. aeruginosa, and the CF microenvironment is a selective force in the evolution of the microbe and its conversion to mucoid phenotype, which is better adapted to survive in these circumstances, thus favoring chronic infection. In this stage, the presence of P. aeruginosa is detectable both on the cell surface and within the mucus [29]. In CF, mucoid forms

increase the production of alginate and Psl polysaccharide, both of which participate in the formation of the biofilm which gives mucoid P. aeruginosa protection against clearance, antibiotics and the immune system [30]. To achieve long-term persistence, the microorganism accumulates a set of mutations in regulatory proteins that lead to the down-regulation of virulence factors that affect motility, transport, quorum sensing, metabolism, DNA repair, and cell division [25]. The mucoid form is associated with the deterioration of CF patients, and with increased morbidity and mortality. Various measures and preventive treatments are therefore carried out in order to retard the transition to the mucoid phenotype [28]. Additionally, more advanced knowledge of the specific interaction of P. aeruginosa with GAGs could facilitate the use of these molecules in infection countermeasures.

Although improvements in therapeutics are being made, currently, there is no cure for CF [1,12,22]. GAGs are employed therapeutically in a variety of diseases, taking advantage of their natural functions or even modifying their structure and activity [31]. In terms of CF, some therapies involving GAGs are applied, including hypertonic saline treatment, which disrupts the interaction of these molecules with peptide LL-37 thereby restoring its antimicrobial activity, as well the use of hyaluronic acid as an anti-inflammatory agent in some treatments [22,31]. The specific characteristics of GAGs may provide new and more efficient therapies to mediate the recurrent infections and inflammatory over-response in CF [31,32]. The long-term treatment of chronic infections with antibiotics leads to the development of resistance, meaning that new anti-infective therapies are urgently needed. The molecular details of the differential involvement of GAGs in bacterial pathogen adhesion to healthy and to CF cells identified in this work may provide new possibilities for treatment options, such as molecules that mimic GAG receptors or specific antibodies, that could play a protective role in the respiratory tract by interfering with certain interactions [22,31,32].

Conflict of interest

None

Authors and contributors

LQ and RG were responsible for the design of the study. FV provided the bacterial clinical isolates. FV and JM carried out the literature search and a review of the manuscript. EV and DR contributed to cell cultures and data analysis. CM and VL performed the experiments, BG analyzed/interpreted the results and drafted the manuscript. All authors have read and approved the final manuscript.

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Legends

Fig 1. Genes involved in HS and CS biosynthesis. The various genes for the enzymes that catalyze the specific reactions are highlighted in red.

Fig. 2. Differential transcription of genes encoding GAG chains. Relative transcript abundance of mRNA for cultured NuLi cells (black bars) and CuFi cells (grey bars) are plotted on a log scale for each gene assayed. (A) Differential transcription of the genes involved in the biosynthesis of the tetrasaccharide glycan linker: Xylosyltransferase I and II (*XYLT1*, *XYLT2*), β-1,4-galactosyltransferase I (*B4GALT7*), β-1,3-galactosyltransferase II (*B3GALT6*), β-1,3-glucuronyltransferase 1-3 (*B3GAT1, B3GAT2, B3GAT3*), xylosylkinase (*FAM20B*), and 2-phosphoxylose phosphatase 1 (*PXYLP1*). (B) Differential transcription of genes involved in the polymerization and modification of CS chains: N-acetylgalactosaminyltransferase 1 and 2 (*CSGALNACT1*, *CSGALNACT2*), chondroitin sulfate synthase 1 and 3 (*CHSY1, CHSY3*), chondroitin polymerizing factor (*CHFP*), chondroitin 4 sulfotransferase 11-14 (*CHST11, CHST12, CHST13, CHST14*), N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (*CHST15*), chondroitin 6 sulfotransferase 3 and 7 (*CHST3, CHST7*), dermatan sulfate epimerase (*DSE*), and uronyl-2-sulfotransferase (*UST*). (C) Differential transcription of genes involved in the polymerization and modification of HS chains: exostosin-like glycosyltransferase 1-3 (*EXTL1, EXTL2, EXTL3*), exostosin glycosyltransferase 1 and 2 (*EXT1, EXT2*), N-deacetylase/N-sulfotransferase 1-4 (*NDST1, NDST2, NDST3, NDST4*), glucuronic acid epimerase (*GLCE*), 2-O-sulfotransferase 1 (*HS2ST1*), 6-O-sulfotransferase 1-3 (*HS6ST1*, HS6ST2, HS6ST3), 3-O-sulfotransferase 1-6 (*HS3ST1, HS3ST2, HS3ST3A1*, HS3*ST3B1*, *HS3ST4, HS3ST5, HS3ST6*), sulfatase 1 and 2 (*SULF1*, *SUL*F2). Statistically significant differences are denoted by * for $p < 0.05$, and *** for $p < 0.001$. Spreads represent standard deviations.
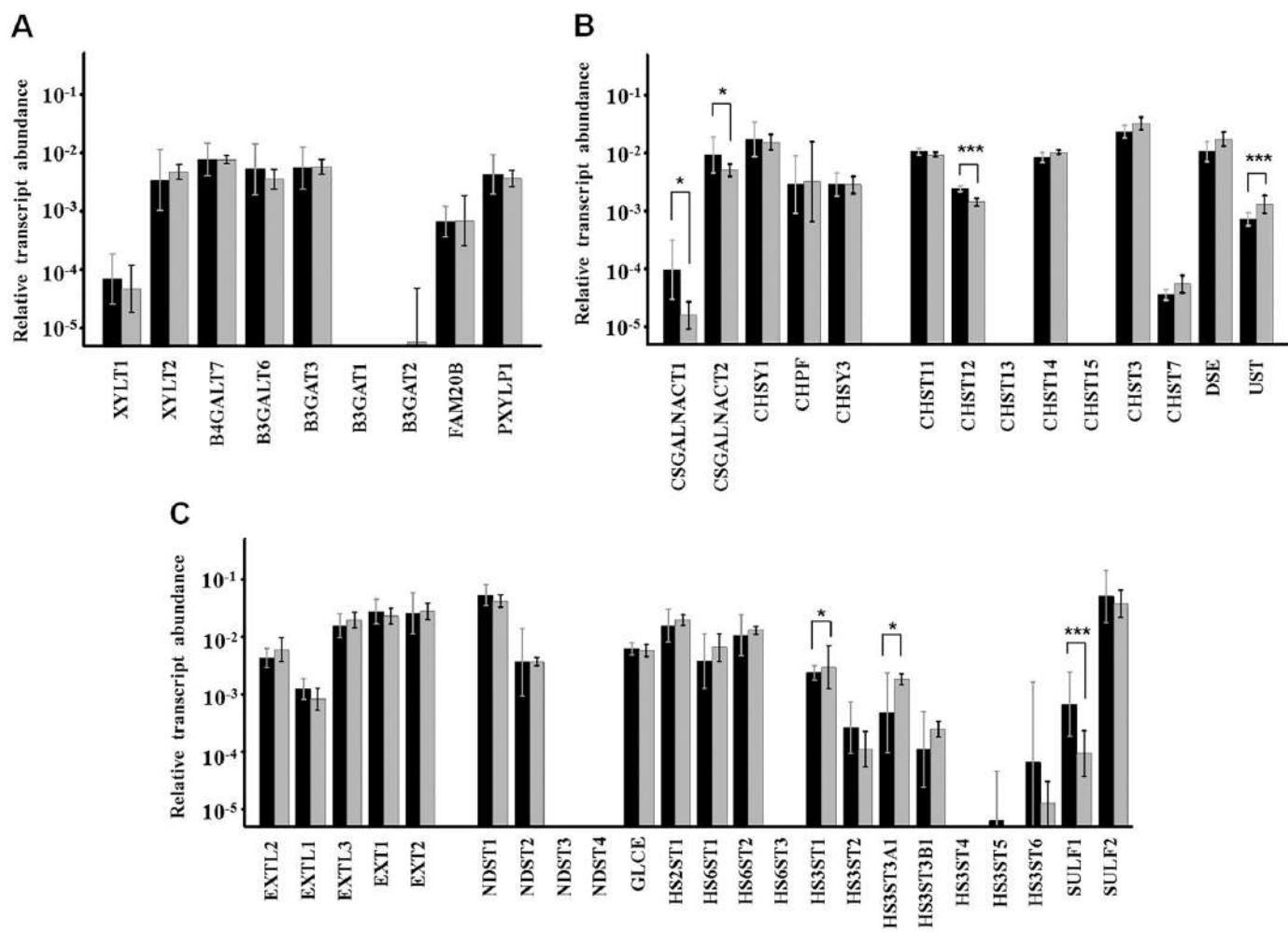
Fig. 3. Effect of enzymatic digestion of cell GAGs on bacterial adherence to lung cells. Effect of pre-treatment of NuLi cells (solid bars) and CuFi cells (striped bars) with GAG lyases, on the binding of clinical isolates (gray bars) and ATCC strains (black bars) of (A) *Staphylococcus aureus*, (B) *Haemophilus influenzae*, (C) *Burkholderia cepacia*, (D) *Pseudomonas aeruginosa* non-mucoid phenotype, and (E) *Pseudomonas aeruginosa*
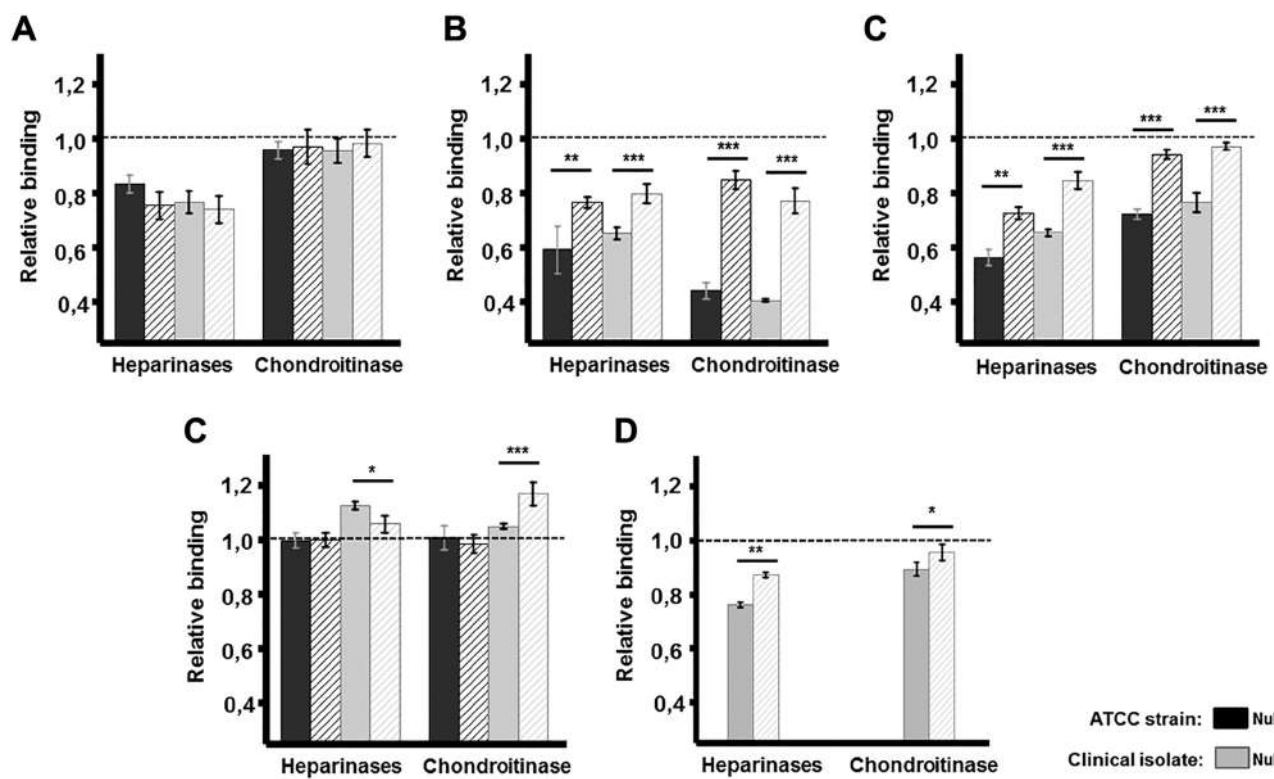
mucoid phenotype. Data were normalized using the adhesion values of bacteria to non-treated cells, which was given the arbitrary value of 1. Statistically significant differences are denoted by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. Spreads represent standard deviations.
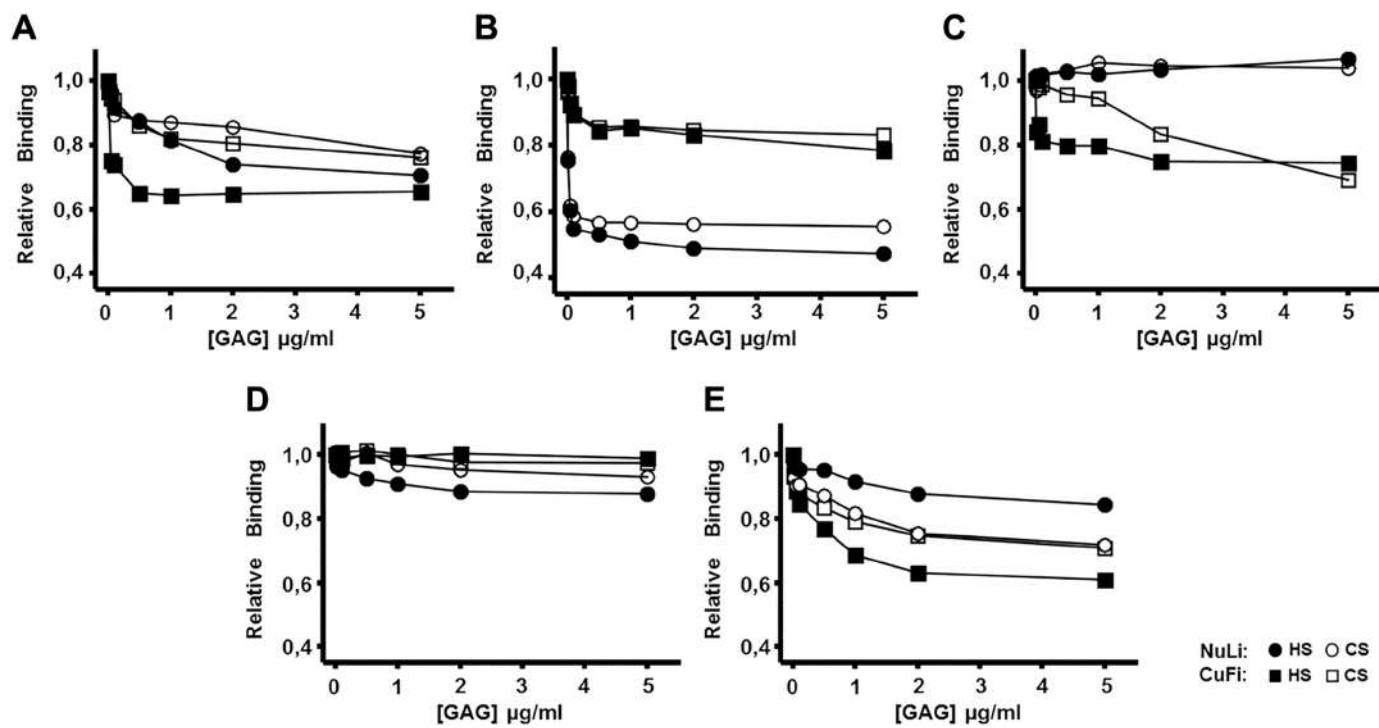
Fig. 4. Inhibition of clinical isolates pathogens attachment to lung cells in the presence of different GAGs. Adhesion of (A) *Staphylococcus aureus*, (B) *Haemophilus influenzae*, (C) *Burkholderia cepacia*, (D) *Pseudomonas aeruginosa* non-mucoid phenotype, and (E) *Pseudomonas aeruginosa* mucoid phenotype to NuLi cells (circles) and CuFi cells (squares) in the presence of HS (black) and a mixture of CSs (white). Data were normalized using the adhesion values of bacteria to non-treated cells, which was given the arbitrary value of 1.
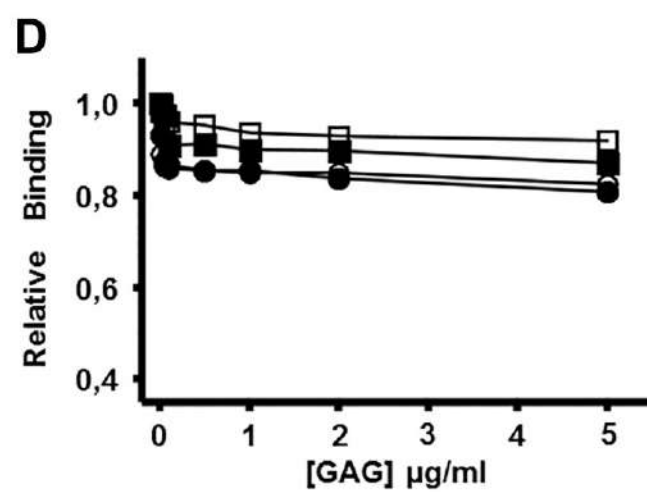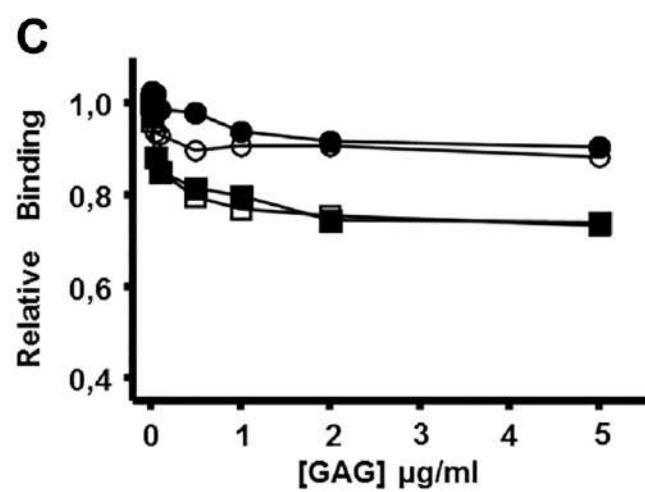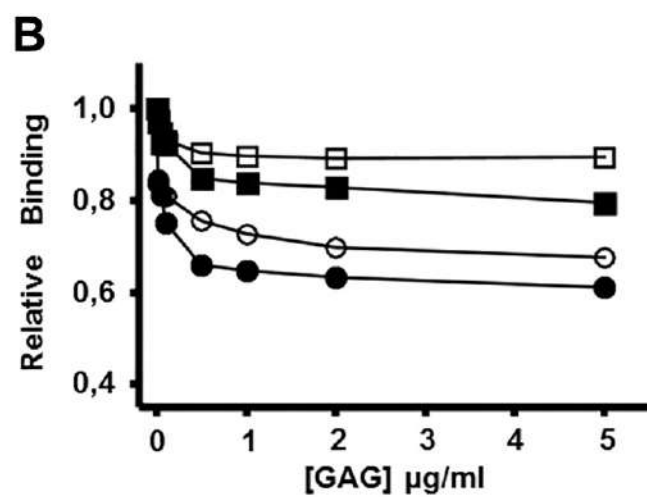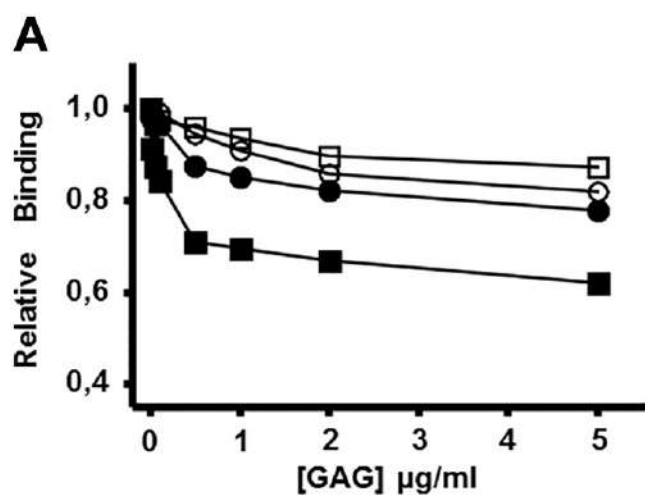
Fig. 5. Inhibition of ATCC strains attachment to lung cells in the presence of different GAGs. Adhesion of (A) *Staphylococcus aureus*, (B) *Haemophilus influenzae*, (C) *Burkholderia cepacia*, and (D) *Pseudomonas aeruginosa* to NuLi cells (circles) and CuFi cells (squares) in the presence of HS (black) and a mixture of CSs (white). Data were normalized using the adhesion values of bacteria to non-treated cells, which was given the arbitrary value of 1.

NuLi: ● HS ○ CS
CuFi: ■ HS □ CS

A

B

C

D

NuLi: ● HS ○ CS
CuFi: ■ HS □ CS