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Effects of heparosan and heparin on the adhesion and biofilm formation of several bacteria *in vitro*

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ABSTRACT

Bacterial adhesion to intestinal mucus and epithelia are important for individual stability of microbial flora. Here we studied the effects of heparosan and heparin on the adhesion and biofilm formation of a *Lactobacillus rhamnosus* strain and several selected pathogens *in vitro*. Results indicated that both heparosan and heparin blocked the adhesion of tested *Escherichia coli*, *Pasteurella multocida*, *Staphylococcus aureus* strains, but did not block the adhesion of *L. rhamnosus* to enterocytes and mucus. Both heparosan and heparin had no significant effect on the biofilm formation of *S. aureus*, while heparin promoted the biofilm formation of *L. rhamnosus*. The increase of biofilm formation might be related to the negative charge of heparin, since no biofilm formation was observed when chitosan was present. This study provides evidence, for the first time, that heparosan can serve as an effective blocker in inhibiting adhesion of pathogens.

1. Introduction

The intestinal habitat of an individual human being contains 300–500 different species. The microbiota keeps a holistic balance for normal health. Both luminal and mucosal microorganisms act in this balance, but the latter is considered more important. Commensal microorganisms usually establish a niche at a safe distance from the host, while most microbial pathogens need to replicate in extreme proximity to the host or even within it (Galan, 2002). Research on the binding and the colonization of pathogens to host cells is of great importance.

Bacterial adhesion to intestinal mucus and epithelia are important for individual stability of microbial flora. Exogenous glycosaminoglycans (GAGs) have shown the ability as competitive inhibitors of bacterial adhesion, among which heparin has been extensively studied. Experiments *in vitro* showed that exogenous heparin or its analog heparan sulfate (HS) specifically reduced the binding of *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Enterococci*, *Staphylococci*, *Streptococc* and *Escherichia coli* to the host cells or immobilized intestinal mucus (Arciola et al., 2003; Fallgren, Andersson, & Ljungh, 2001; Frick, Schmidtchen, & Sjobring, 2003; Gu, Wang, Guo, & Zen, 2008; Henry-Stanley, Hess, Erickson, Garni, & Wells, 2003; Hess, Henry-Stanley, Erlandsen, & Wells, 2006). Exogenous heparin may block the bacterial exploitation of host GAGs and inhibit the infections to the host. Heparin or HS has a potential to be a broad-spectrum anti-infection agent (Lever & Page, 2002).

Heparosan (*N*-acetylheparosan, or desulfoheparin) is constructed by the repeating disaccharide unit $\rightarrow 4GlcUA\beta 1 \rightarrow 4GlcNAc\alpha 1 \rightarrow$ as backbone of the polysaccharide found in the capsule of certain bacteria. It has also been found in animals from hydra to vertebrates as the biosynthetic precursor of heparin or HS (Sismey-Ragatz et al., 2007). Heparosan has the similar sugar backbone with heparin or HS, except the polymer is nonsulfated, and there is no epimerization of GlcUA to IdoUA (DeAngelis, Gunay, Toida, Mao, & Linhardt, 2002). There are two kinds of bacteria that have been found with the capsule polysaccharide of heparosan, *E. coli* K5 and *Pasteurella multocida* type D. The discovery of heparosan synthase in bacteria makes the production of heparin or HS through fermentation followed by chemical or biological modification become possible. Many studies have shown the effects of heparosan and its analogs on antiangiogenic activity, modulating the fibroblast growth factor signaling and inhibiting the infection of virus (Maddineni et al., 2006; Presta et al., 2005; Rusnati et al., 2009). But the impact of heparosan on the adhesion of bacteria remains unknown. Here we studied the effects of heparosan on the adhesion of several bacteria to enterocyte and mucus binding, including a safe probiotic bacterium, *Lactobacillus rhamnosus* GG (LGG) (Huang, He, Zhou, Wu, & Jong, 2009), and several pathogenic strains, as well as on the biofilm formation of LGG and *Staphylococcus aureus*. The data would be compared with that of heparin.

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2. Materials and methods

2.1. GAGs

Heparin (unfractionated heparin), porcine intestinal mucosa, was purchased from Hebei Changshan Biochemical Pharmaceutical Co., Ltd. (Zhengding, Shijiazhuang, China). The heparosan was prepared as described below. Type D P-934 *P. multocida* (*P. multocida* sunsp. *multocida* ATCC® 12948™) was grown in BHI. Growth at 37 °C with wild shaking for 48 h resulted in dense growth and luxurious capsule production. The heparosan was isolated from the fermentation broth with a modified method (DeAngelis & Padgett-McCue, 2000). Briefly, a 1% solution of cetylpyridinium chloride (CPC) was added to the supernatant fluid containing heparosan, whereupon heparosan precipitated as cetylpyridinium–heparosan complex. The precipitate was dissolved in 0.3 M NaCl solution. Then, three volumes of ethanol were added to the solution to precipitate heparosan and after centrifugation, the precipitate was dissolved in distilled water. The resuspension in salt solution, followed by ethanol precipitation, was repeated twice. The described protocol furnished heparosan with above 98% purity as evaluated by HPLC and NMR spectroscopy. The weight-averaged molecular weight was 150 kDa, evaluated by size-exclusion chromatography-multiangle laser light scattering (SEC-MALLS) analysis.

2.2. Bacterial strains and growth conditions

LGG (*L. rhamnosus* CGMCC 1.3724) was obtained from China General Microbiological Culture Collection Center. Strains of *entero-pathogenic E. coli* (EPEC), *S. aureus* and *Salmonella* were obtained from the Department of Clinical Microbiology, Shandong University. The recombinant EPEC that expresses green fluorescent protein is a kind gift of Professor Fengshan Wang (School of Pharmaceutical Sciences, Shandong University, China) and the *P. multocida* was obtained from ATCC. LGG was incubated overnight in MRS broth anaerobically, strain of *P. multocida* was incubated overnight in BHI broth, while strains of EPEC, recombinant EPEC, *S. aureus* and *Salmonella* were incubated overnight in LB broth individually. For the determination of bacterial concentration, the method of serial dilution followed by viable plate counts on appropriate agar media was used.

2.3. Enterocytes binding/internalization

HT-29 human colon carcinoma cell line, which is known to abundantly express cell surface HS, was used in this study. HT-29 enterocytes, obtained from Shandong Academy of Medical Science were cultivated in RPMI-1640 (Thermo Fisher Scientific) at 37 °C supplemented with 10% fetal bovine serum (FBS), 5% CO_2. To obtain a mature, confluent enterocyte layer, HT-29 cells were cultivated for 10 days in 24-well plates. Enterocyte viability was consistently >95%, verified by staining with 0.36% trypan blue. Binding/internalization assaying was based on the procedures of Hess et al. (2006). The plates were washed with PBS before bacterial binding. Bacterial cells were washed and resuspended to 10^8 CFU/ml with RPMI-1640 without FBS. Bacterial suspensions were allowed 1 h for binding to enterocytes at 37 °C in the presence or absence of heparosan/heparin. For competition binding assay, EPEC and LGG suspensions were added simultaneously both with and without heparosan/heparin present. For displacement and resistance binding assay, EPEC and LGG suspensions were added sequentially with both sequences used. After binding, the plates were washed with PBS for five times, ensuring that any remaining non-adherent bacteria would be <1% of the total adherent CFUs (pilot experiments confirmed). Then enterocytes were lysed with 0.25% trypsin–0.02% EDTA for 10 min and viable bacteria (the adherent and internalized bacteria) were quantified by serial dilution and viable plate counts. The average in triplicate wells was considered one assay value, repeated on three separate days.

2.4. Mucus binding

Mucus binding was performed with a modified method (Gusils, Morata, & Gonzalez, 2004). Briefly, 300 μl of porcine stomach mucin type III (Sigma) aqueous solution (50 mg/ml) or bovine serum albumin (BSA) aqueous solution (50 mg/ml) was immobilized in 48-well polystyrene tissue culture plates for 48 h at 4 °C. The plates were washed twice with HEPES-Hanks buffer. Fresh bacterial cultures were washed with PBS and resuspended in HEPE-Hanks buffer with or without heparosan/heparin (50 μg/ml). The suspensions were then applied to the immobilized mucin, BSA-coated, or uncoated plates and allowed 1 h at 37 °C for sufficient binding. Then all the plates were washed three times with HEPES-Hanks buffer and 0.5 ml PBS supplemented with 1% Triton-X-100 was added to each well for 15 min. Bacterial concentrations in each well were determined individually.

2.5. Biofilm formation

A 200 μl of bacterial suspension (~10^7 CFU/ml) was added to each well in microtiter plates and allowed 24 h at 37 °C for biofilm formation in the presence or absence of heparosan/heparin (50 μg/ml). The plates were washed with PBS and then stained for 30 min with 200 μl 0.1% crystal violet in an isopropanol–methanol–PBS solution (1:1:18 [vol/vol/vol]). Excessive dye solutions were removed and the plates were washed with 200 μl distilled water for three times. After air-dried for 30 min, a volume of 200 μl 30% acetic acid was added to each well. The absorbance was measured at 570 nm (Peeters, Nelis, & Coenye, 2008). Each condition was tested in at least three independent experiments, each with eight biological replicates. Sterile medium was included as negative control. Chitosan was tested for comparison as well.

2.6. Statistical analysis

Bacterial numbers were converted to $\log_{10}$ prior to statistical analysis, and significance was set at $P \leq 0.05$. Differences were analyzed by one-way analysis of variance followed by Fisher's test. The SPSS 16.0 statistical package was used for analysis.

3. Results

3.1. Effects of heparosan/heparin on the enterocyte binding/internalization of LGG and selected pathogens

We first examined the effects of different concentrations of heparosan and heparin on the adhesion of EPEC to cultured HT-29 cells. As shown in Fig. 1A, adhesion of EPEC to HT-29 cell surface was shown to decrease in a dose-dependent fashion. The concentration of 50 μg/ml for heparosan and heparin, with efficient ability of anti-adhesion of selected pathogens was used in other assays. The data showed that heparosan and heparin inhibited EPEC, *P. multocida* and *S. aureus* adherence individually, but had no significant effect on the binding of tested strains of LGG and *Salmonella* (Fig. 1). There was no obvious difference between the inhibition effects of heparosan and heparin.

To further explore if there was a synergy effect of heparosan and LGG in the anti-adhesion of EPEC, the competition, displacement and resistance binding assays were performed. Results showed that heparosan contributed little to the anti-adhesion of EPEC in the presence of high concentration of LGG while helped when the

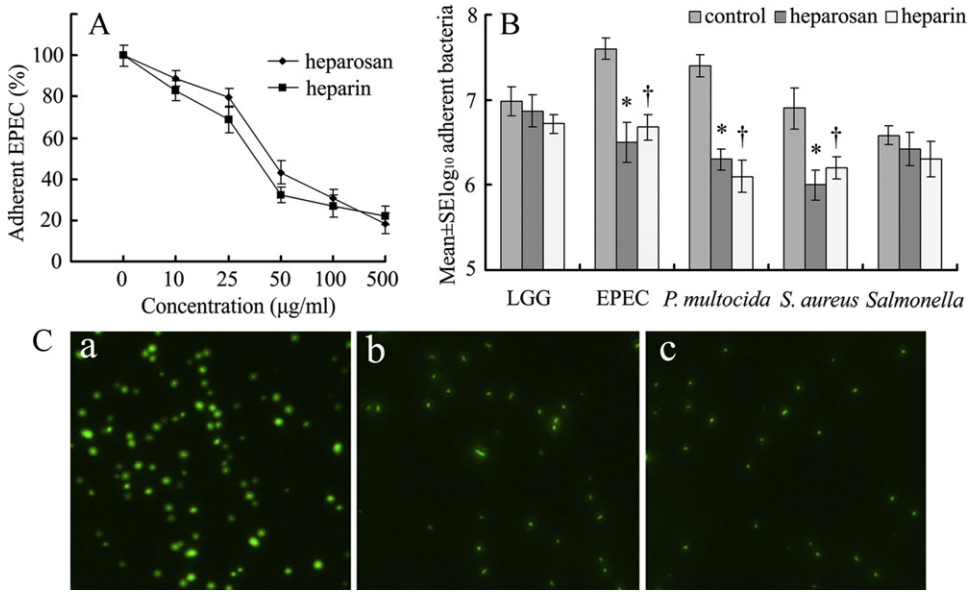

Fig. 1. Effects of heparosan and heparin on the adhesion of selected bacteria to HT-29 enterocytes. (A) Adhesion of EPEC to HT-29 cells in the presence of various concentration of heparosan and heparin. (B) The bacterial adhesion to HT-29 cells in the presence of 50 μg/ml heparosan/heparin, *, † decreased compared to the control, $P \leq 0.05$. (C) The adhesion of recombinant EPEC to HT-29 cells: (a) control; (b) heparosan; (c) heparin.

concentration of LGG was low (Fig. 2). There was no significant difference in displacement assays, indicating that heparosan mainly interfered with the binding process of EPEC to enterocytes without causing detach of attached bacteria from enterocytes. Heparin was shown with the similar effects (Fig. 2).

3.2. Effects of heparosan/heparin on the mucus binding of LGG and EPEC

The epithelial cells in gastrointestinal tract are covered by a relatively thick layer of mucus, which consists of mucin, many small associated proteins, glycoproteins, lipids, and glycolipids. Bacterial adhesion to mucus is important for individual stability of microbial flora. We tested the effects of heparosan and heparin on the mucus binding of LGG and EPEC. Results showed heparosan and heparin reduced mucus binding of EPEC to immobilized mucin and BSA, but had no effect on the binding to polystyrene. As for LGG, all types of binding were not affected (Fig. 3). There was no obvious difference between the inhibition effect of heparosan and heparin.

3.3. Effects of heparosan/heparin on the biofilm formation of LGG and S. aureus

It is widely accepted that the majority of bacterial infection involve microbial growth as a film. Therefore, we tested the biofilm formation of LGG and *S. aureus* in the presence of heparosan and heparin. Results showed heparosan and heparin had no significant effect on the biofilm formation of *S. aureus*, while heparin promoted the biofilm formation of LGG with above 30% increase (Fig. 4). This increase was partially related to the negative charges of heparin, since there was no biofilm formation when chitosan was present.

4. Discussion

Microbial infection is a complicated process largely dependent on the host-microbe relationship. Usually, adhesion is the first step leading to colonization and subsequent infection (Sava et al., 2009). Various cellular (surface) components on eukaryotic cells

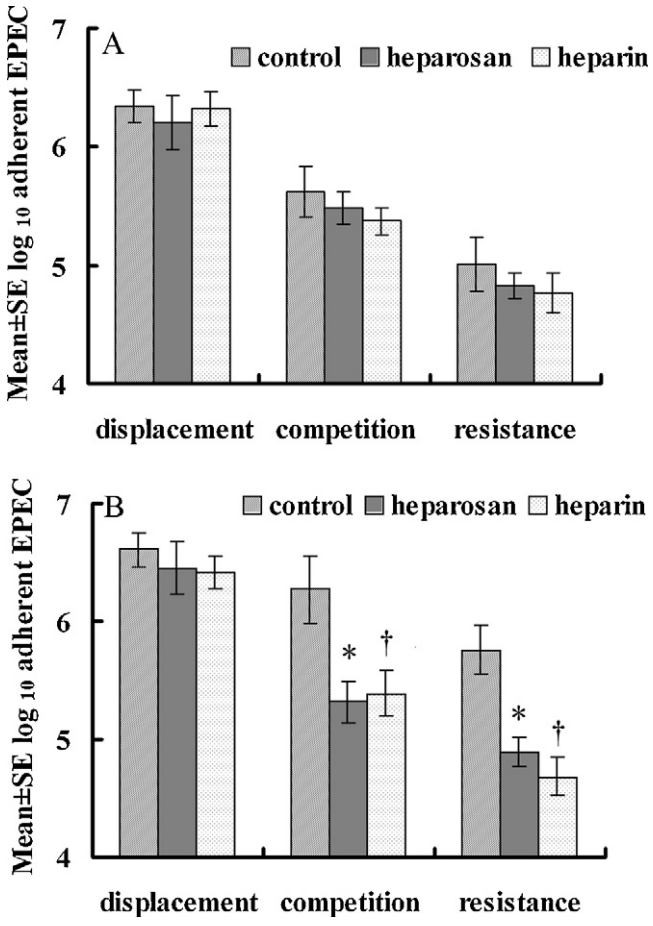

Fig. 2. Displacement, competition and resistance binding assays of LGG against EPEC by HT-29 enterocytes, in the presence of 50 μg/ml heparosan/heparin. (A) 5×10^8 CFU/ml and (B) 5×10^6 CFU/ml LGG were tested. For EPEC, *, † decreased compared to the control, $P \leq 0.05$.

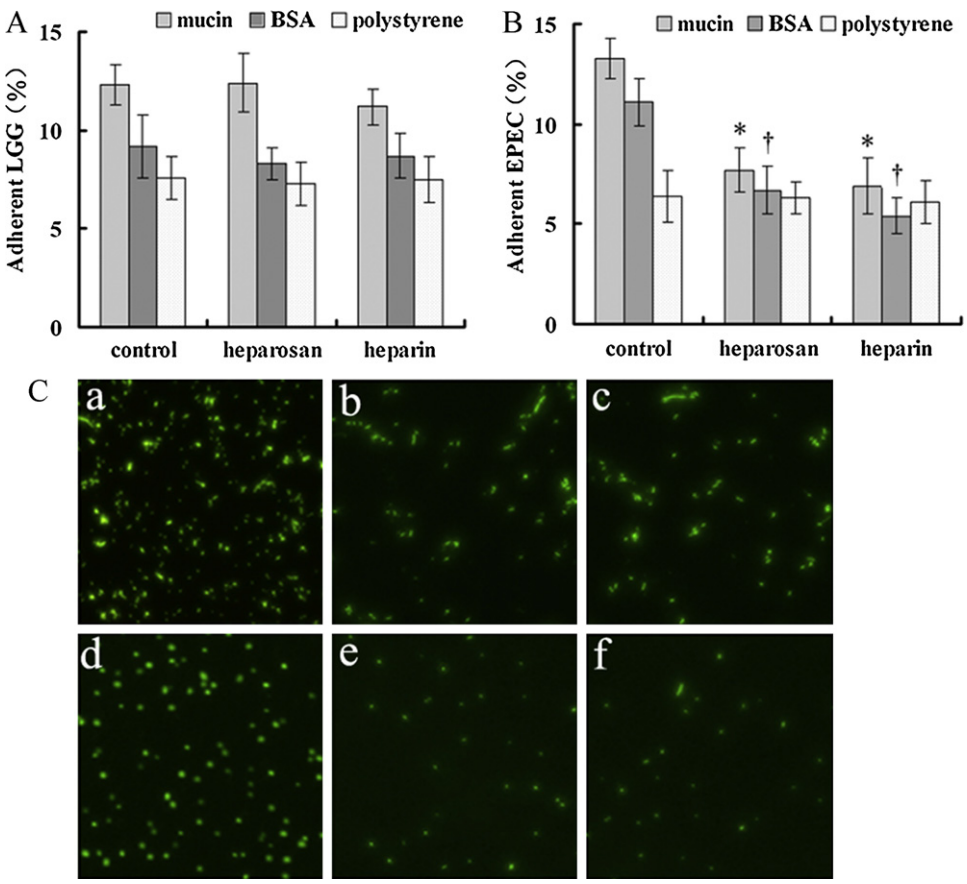

Fig. 3. The adhesion of LGG and EPEC by mucin, BSA and polystyrene, in the presence of 50 μg/ml heparosan/heparin. (A) LGG and (B) EPEC, bacterial adhesion was represented as the percentage of total applied bacteria, Data represented means ± SE of three independent experiments performed in triplicate. *, †Decreased compared to the control, $P \leq 0.05$. (C) The adhesion of recombinant EPEC to mucin (a–c) and BSA (d–f): (a and d) control; (b and e) heparosan; (c and f) heparin.

and tissues have been identified as targets for adhesion by microorganisms and many of them are characterized as proteoglycans. Exogenous GAGs have shown the ability as competitive inhibitors of bacterial adhesion, among which heparin has been studied most. Heparosan, as the biosynthetic precursor of heparin, has the similar sugar backbone with heparin, which also affects the adhesion of several pathogenic strains as shown in this paper.

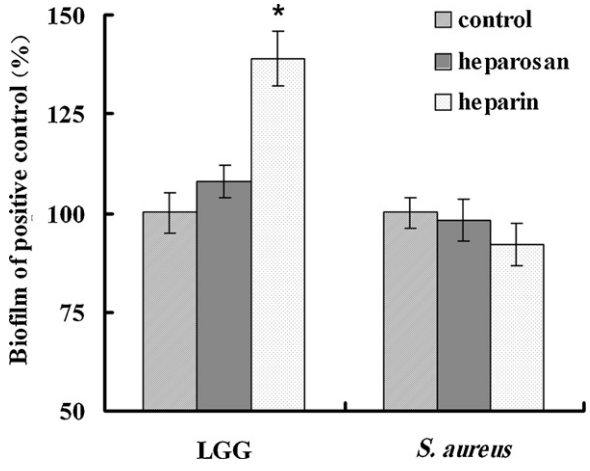

Fig. 4. Biofilm formation of LGG and *S. aureus*, in the presence of 50 μg/ml heparosan/heparin. Biofilm formed were measured as absorbance of crystal violet at 570 nm, compared to that of LGG/*S. aureus* in medium which were taken as 100%. *Increased compared to the control, $P \leq 0.05$. Data represented means ± SE of three independent experiments performed in triplicate.

Gastrointestinal microbial infection is traditionally treated with broad-spectrum antibiotics. Except for the problem of drug resistance, the application of antibiotics exerts suppression to the whole microbiota, which may break the holistic balance. Under specific conditions, some antibiotics even promote the biofilm development of certain pathogens (Hess, Henry-Stanley, & Wells, 2011). Unlike commonly used broad-spectrum antibiotics, we found that heparosan and heparin did not hamper the adhesion of tested LGG strain to enterocytes or mucus (Figs. 1B and 3A). When high density of LGG cells was present, inhibition of EPEC by additional heparosan/heparin was not apparent (Fig. 2A). However, better results from competition and resistance binding assays were achieved with the presence of heparosan/heparin when the density of LGG cells was low (Fig. 2B). Considering that the viability of probiotic strains is usually lowered in the low pH environments of stomach, heparosan/heparin as supplementary is more meaningful. It seemed that heparosan/heparin only worked well for the pre-treatment of pathogenic bacteria before binding, considering the poor performance in displacement binding assays (Fig. 2). Thus heparosan/heparin could be potentially used as supplementary to probiotics for the prophylaxis of the onset of infections. Furthermore, both heparosan and heparin showed no significant effect on the biofilm formation of *S. aureus*, while heparin facilitated the colonization of LGG by prompting biofilm formation (Fig. 4). This facilitation might be due to the high negative charge density of heparin, since no biofilm formation was observed for positively charged chitosan. This was also confirmed with our results that heparosan, as nonsulfated, with low negative charge density, had no significant effect on the biofilm formation of LGG.

There were no obvious differences between the inhibition effects of heparosan and heparin on the adhesion of selected pathogens to enterocyte and mucus binding. Heparosan is superior to heparin which has the risk of bleeding due to its anticoagulation effect. Especially, heparosan can be largely synthesized by bacteria (Wang et al., 2010) and sulfated to different analogs with higher negative charge density (Zhang et al., 2008) that may improve prompting effect on the biofilm formation of LGG. Our finding, with further study, may offer the possibility of exploiting heparosan and its analogs for novel pharmacological treatments for gastrointestinal microbial infection.

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