Fibronectin binding of *Lactobacillus* species isolated from women with and without bacterial vaginosis

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Summary. Lactobacilli isolated from the vaginas of healthy women (39 strains) and from the vaginal discharge of women with bacterial vaginosis (15 strains) were investigated for their binding to ^{125}I -fibronectin. Nine of the 54 strains bound fibronectin at pH 7.2. The binding capacity of these nine strains was about the same as that observed with *Staphylococcus aureus* Cowan 1. The binding was specific; an excess of unlabelled fibronectin or its amino-terminal 29-kDa fragment effectively competed for binding, whereas bovine serum albumin, human IgG and orosomucoid did not. Incubation of lactobacilli with fibronectin for different periods revealed a time-dependent increase in binding. Lowering the pH to 4.0 increased the binding capacity of all of the lactobacilli tested; binding occurred with strains that had previously failed to bind at pH 7.2. The increased binding of lactobacilli to fibronectin at a low pH may play a role in the maintenance of the ecological balance of the vagina.

Introduction

Bacterial vaginosis is a syndrome characterised by a homogeneous thin discharge with a pH value > 4.7 , a fishy amine odour and the presence of clue cells.¹ Besides these clinical signs, a significant decrease of the normal *Lactobacillus* flora of the vagina can be seen with a simultaneously increased concentration of a mixed anaerobic flora including *Mobiluncus* spp, together with *Gardnerella vaginalis* and mycoplasmas.^{2,3} The mechanisms that control the composition of the vaginal microflora under different circumstances are only partly understood. The low pH of the vaginal fluid in healthy premenopausal women is probably maintained by the lactobacilli, that metabolise the glycogen in the vaginal epithelium through glucose to lactic acid.⁴ The antibiosis between lactobacilli and other bacteria,⁵ due in part to H_2O_2 ,⁶ or bacteriocin⁷ production, and the adherence of the different bacteria to vaginal epithelial cells,^{8–10} are only some factors that may influence the ecological balance of the vagina.

Fibronectin is a high-mol. wt glycoprotein found in soluble form in plasma and in different body fluids such as the vaginal fluid.^{11,12} Insoluble or matrix fibronectin is a web of polymerised fibronectin that coats cell surfaces, basement membranes and mucosal or cutaneous surfaces.¹³ Fibronectin has several adhesive functions and may reasonably be assumed to be a central and indispensable component in a number of

physiologically diverse systems. Fibronectin may play a dual role in host-parasite interactions.¹⁴ Favourably for the host, fibronectin provides an ideal foundation material to which structural macromolecules or cells such as members of the indigenous flora can adhere. In this way, it may enhance the attachment of symbiotic organisms to mucosal surfaces, thus establishing the protective normal bacterial flora. On the other hand, the binding of pathogenic bacteria to fibronectin can be the first step in the infective process. Fibronectin binding has been proposed as the initial step in infection caused by several pathogens, e.g., *Staphylococcus aureus*,¹⁵ β -haemolytic streptococci,^{16,17} *Escherichia coli*,¹⁸ *Salmonella enteritidis*¹⁹ and *Treponema pallidum*.²⁰ There are only limited data about the fibronectin binding of the members of the normal mucosal flora.

These considerations prompted us to perform a systematic screening and characterisation of the fibronectin binding of lactobacilli isolated from the vaginas of healthy women and from others suffering from bacterial vaginosis.

Materials and methods

Bacteria

Studies were performed on 54 *Lactobacillus* isolates from the vaginal fluid of healthy women (39 strains) and from the vaginal discharge of women with bacterial vaginosis (15 strains). The biochemical

characterisation of the lactobacilli was with the API 50 CH kit (bioMérieux). Fibronectin binding was also tested with four additional *Lactobacillus* reference strains from the culture collection of the University of Göteborg (*L. acidophilus* CCUG 5917, *L. fermentum* CCUG 21453, *L. casei* CCUG 18011 and *L. delbrueckii* CCUG 19776) and one *L. acidophilus* strain isolated from the Lactinette capsule (Biologa AB, Sweden), which is recommended for use in cases of bacterial vaginosis to re-establish the dominance of the lactobacillary flora. *S. aureus* strain Cowan 1 was selected as a positive control strain for fibronectin binding. Before use, the *Lactobacillus* strains were cultured on Rogosa Agar (Difco) plates at 37°C in an anaerobic environment (GasPak system; BBL) for 48 h. Bacteria used to measure the binding of ^{125}I -fibronectin were harvested in 5 ml of phosphate-buffered saline (PBS; pH 7.2), centrifuged at 1500 *g* for 10 min and washed once with the same buffer. The final density of the suspension was adjusted to 10^{10} cfu/ml. This bacterial suspension could be stored at -20°C without changes in the fibronectin binding capacity.

^{125}I -fibronectin binding

Labelled fibronectin was a gift from M. Lindberg (Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden). Fibronectin was isolated from bovine plasma and labelled with ^{125}I by the chloramine-T method.²¹ The binding of ^{125}I -fibronectin to the bacteria was determined by a modification of the procedure described previously.²² Briefly, bacterial suspensions stored at -20°C were diluted 10-fold in PBS with Tween 80 0.1% v/v. To 2.9 ml of this suspension, ^{125}I -fibronectin 2×10^4 cpm in 0.1 ml (specific activity 8.2 $\mu\text{Ci}/\mu\text{g}$) was added. Tubes containing reaction mixtures were incubated with rotation for 2 h at room temperature. The samples were then centrifuged at 1500 *g* for 20 min. The supernates were aspirated and the radioactivity associated with the pellet was measured in a γ -counter (LKB Wallac Clinigamma 127, Turku, Finland). The analyses were always made in duplicate. The binding of fibronectin was expressed as a percentage of the total activity after subtraction of the background values. The fibronectin binding of lactobacilli at different pH values was tested in the same way. Citrate-phosphate buffer of pH 7.0, 6.0, 5.0 and 4.0 was used, or phosphate buffer of pH 8.0 supplemented with Tween 80 0.1% v/v. Control tubes containing buffer at different pH values were also used without bacteria and with the same amount of ^{125}I -fibronectin.

Inhibition of ^{125}I -fibronectin binding

Unlabelled fibronectin isolated from bovine plasma (Extraco, Sweden), the 29-kDa amino-terminal fragment of human fibronectin, isolated as described by Fröman *et al.*,¹⁸ immunoglobulin G (IgG) (KabiVitrum, Sweden), orosomucoid (Waddie,

Edinburgh) and bovine serum albumin (Sigma), each dissolved in 0.2 M phosphate buffer (pH 7.2), were used to study the inhibition of ^{125}I -fibronectin binding. Competitive inhibition assays were performed in the same way as the binding assays except that 10 μg or 100 μg of unlabelled fibronectin, its 29-kDa fragment or one of the other proteins was added to the test-tubes before the addition of ^{125}I -fibronectin. The extent of inhibition was expressed as a percentage of the total binding of ^{125}I -fibronectin to the bacteria without other proteins.

Ultrasound treatment of lactobacilli

Diluted suspensions (10-fold in PBS+Tween 80 0.1% or citrate-phosphate buffer, pH 4.0, +Tween 80 0.1%) of *L. jensenii* strain 34 and *L. acidophilus* strain 71 were treated with ultrasound (Bransonic, Model B-2200, Dantury, CT, USA) for 1, 3, 5 or 10 min before the addition of ^{125}I -fibronectin. After incubation for 2 h, the binding of fibronectin was determined as already described; 100 μl of the supernate of the ultrasound-treated (10 min) undiluted cell suspensions was used to inhibit the binding of ^{125}I -fibronectin.

Results

Of 54 isolates of lactobacilli tested in the ^{125}I -fibronectin binding assay at pH 7.2, nine strains, all from healthy women, bound fibronectin, whereas 45 strains (30 from healthy women and 15 from patients with bacterial vaginosis) did not. When the fibronectin binding values were compared with that of *S. aureus* Cowan 1, which was taken as 100%, the nine positive *Lactobacillus* strains could be ranged in three categories—high, medium and low binders (fig. 1). All four *Lactobacillus* strains from the type culture collection of the University of Göteborg and the *L. acidophilus* isolate from the Lactinett capsule did not bind fibronectin at pH 7.2.

Specificity of ^{125}I -fibronectin binding was tested by competitive inhibition experiments with a representative strain from each of the three categories. The ^{125}I -fibronectin binding was almost completely inhibited in all three strains by 100 μg of unlabelled fibronectin (table I). The 29-kDa fragment of fibronectin inhibited the binding of ^{125}I -fibronectin to almost the same degree as intact fibronectin, which strongly indicates that the lactobacilli interact with the amino-terminal domain of the fibronectin molecule. No marked inhibition of fibronectin binding was observed with human IgG, orosomucoid or bovine serum albumin.

The fibronectin binding of the nine *Lactobacillus* strains with different binding capacities and that of 15 *Lactobacillus* strains which proved to be non-binders at pH 7.2 was determined at different pH values. A marked increase in the binding capacity was observed in all groups of lactobacilli tested which paralleled the

Fig. 1. Relative ¹²⁵I-fibronectin binding of 54 clinical isolates of lactobacilli at pH 7.2; binding by *S. aureus* strain Cowan 1 was taken as 100%.

Table I. Specificity of ¹²⁵I-fibronectin binding by *L. jensenii* (high), *L. acidophilus* (medium) and *L. casei* (low) at pH 7.2

Species and strain no.	Amount of protein added (μg)	Percentage of ¹²⁵ I-fibronectin bound by bacteria in the presence of				
		bovine fibronectin	29-kDa fragment	human IgG	orosomucoid	bovine albumin
<i>L. jensenii</i> strain 34	10	17	24	90	89	84
	100	10	20	78	86	78
<i>L. acidophilus</i> strain 71	10	13	20	93	94	100
	100	6	18	90	85	100
<i>L. casei</i> strain 33	10	10	13	100	92	99
	100	8	10	94	86	96

* Percentage of total binding by the bacteria incubated without the unlabelled protein.

decrease in pH. At pH 7.0, the four categories of strains could easily be differentiated, i.e., high, medium and low binders and non-binders. However, at pH 4.0 all strains displayed a markedly higher binding of ¹²⁵I-fibronectin, with no differences between the members of the originally established categories. Even *Lactobacillus* strains which proved to be non-binders at pH 7.0 (< 1% of total radioactivity added) exhibited a much higher ¹²⁵I-fibronectin binding than that of the reference strain *S. aureus* Cowan 1 at pH 7.0. With increase of the pH to 8.0, the binding capacity of the lactobacilli was less than that observed at pH 7.0 in every group (table II). We also tested the influence of the pH value on the binding capacity of *S. aureus* Cowan 1. An increase in its ¹²⁵I-fibronectin binding was likewise observed in parallel with the decrease of the pH (from 6.5% of total radioactivity at pH 7.2 to 16% at pH 4.0).

Inhibition experiments with unlabelled fibronectin and its 29-kDa fragment were done to confirm the specificity of the binding of labelled fibronectin at pH 4.0. A representative strain from each of the four

categories already described was used (table III). For all four strains, inhibition of ¹²⁵I-fibronectin binding was observed in the presence of 100 μg of unlabelled fibronectin but this proved to be less than that measured at pH 7.2. The 29-kDa fragment of fibronectin displayed a slightly greater inhibition. However, human IgG, orosomucoid and bovine serum albumin did not inhibit the binding of the labelled fibronectin at pH 4.0.

The kinetics of ¹²⁵I-fibronectin binding at different pH values was investigated with *L. jensenii* strain 14, a high-binding strain. The binding was found to be time-dependent, with a maximum after incubation for 2 h at pH 7.0 (fig. 2). At pH 4.0, the binding of fibronectin to *L. jensenii* strain 14 was very rapid and the percentage of protein bound after incubation for 15 min did not change appreciably during further incubation.

Cells of *L. jensenii* strain 34 and *L. acidophilus* strain 71 were treated by ultrasound for different periods. Ultrasound treatment for 10 min before adding the ¹²⁵I-labelled fibronectin to the reaction mixture completely abolished the binding of fibronectin at pH 7.2,

Table II. Effect of pH on ^{125}I -fibronectin binding of lactobacilli

Species and strain no.	Capacity of binding at pH 7.0	Percentage of ^{125}I -fibronectin binding at pH*				
		8.0	7.0	6.0	5.0	4.0
<i>L. jensenii</i> strain 34	high	5	11	16	20	32
<i>L. acidophilus</i> strain 71	medium	3	5	11	14	28
<i>L. casei</i> strain 33	low	< 1	2	5	10	29
<i>L. fermentum</i> strain 56	"non"	< 1	< 1	4	18	34

* Binding of ^{125}I -fibronectin expressed as a percentage of the total activity after subtracting the background values.

Table III. Specificity of ^{125}I -fibronectin-binding of different strains of lactobacilli at pH 4.0

Species and strain no.	Amount of protein added (μg)	Percentage of ^{125}I -fibronectin bound by bacteria in the presence of*				
		bovine fibronectin	29-kDa fragment	human IgG	orosomucoid	bovine albumin
<i>L. jensenii</i> strain 34	10	71	62	92	90	94
	100	60	55	83	85	82
<i>L. acidophilus</i> strain 71	10	78	54	100	95	99
	100	61	53	95	92	98
<i>L. casei</i> strain 33	10	72	58	100	92	98
	100	58	53	91	87	90
<i>L. fermentum</i> strain 56	10	75	60	100	90	97
	100	55	57	92	82	92

* Percentage of total binding by the bacteria incubated without the unlabelled protein.

Fig. 2. Time curve of ^{125}I -fibronectin binding by *L. jensenii* strain 14 at pH 7.2 (■—■) and pH 4.0 (●—●).

whereas < 5 min treatment did not influence binding. The binding of fibronectin by untreated cells was not inhibited by adding 100 μl of the supernate of the ultrasound-treated undiluted cells to the reaction mixture.

Discussion

Lactobacilli compose the predominant vaginal flora of premenopausal women without apparent genital infections.² It has been postulated that lactobacilli are

responsible for the low vaginal pH (3.3–4.5), by production of lactic acid from glycogen.⁴ Such a low pH value would be expected to inhibit the growth of non-acidophilic organisms, e.g., mixed anaerobic flora seen in bacterial vaginosis or other aerobic pathogens, while stimulating the growth of acidophilic organisms such as lactobacilli.

The adherence of lactobacilli to washed vaginal epithelial cells *in vitro* has been studied by several authors.^{8–10} Pathogens such as group B streptococci, *Neisseria gonorrhoeae* and *G. vaginalis* were reported by Mårdh and Weström⁸ to adhere better than lactobacilli. On the other hand, Sobel *et al.*⁹ reported that lactobacilli and some vaginal pathogens adhered equally. Wood *et al.*¹⁰ investigated the adherence of lactobacilli isolated from healthy women and could not detect any differences in their adherence to washed vaginal epithelial cells *in vitro* at pH 4.8, 6.0 and 7.2.

Fibronectin is a large glycoprotein of c. 440 kDa; it is widely distributed in different body fluids and can be secreted on to and coat mucosal surfaces.^{11,13,14} Antonas *et al.*²³ reported fibronectin levels as high as 90–140 $\mu\text{g}/\text{ml}$ in the vaginal fluid during menses, whereas the levels were low (3–5 $\mu\text{g}/\text{ml}$) at the end of the menstrual cycle. Cohen *et al.*⁷ measured the fibronectin content of the vaginal fluid of patients with

acute gonococcal cervicitis and that of non-infected control women. The concentrations of fibronectin were 0.2–30 µg/ml, without significant differences between the patients and healthy women.

In the present study, marked differences were observed in the capacities of *Lactobacillus* isolates to bind ¹²⁵I-labelled fibronectin when this was tested at different pH values. Only nine of 54 vaginal isolates (all from the vaginal fluid of healthy women) bound ¹²⁵I-fibronectin at pH 7.2. This binding should be considered to be specific, because it was inhibited by unlabelled fibronectin or its 29-kDa amino-terminal fragment. The inhibition of binding of ¹²⁵I-fibronectin by the 29-kDa fragment indicates that the lactobacilli bind to the amino-terminal domain of the fibronectin molecule, as do *S. aureus* strains^{15, 16, 22} or streptococci.^{16, 17} On lowering the pH from 8.0 to 4.0, increased binding of fibronectin was observed with all of these nine *Lactobacillus* isolates. Fifteen further isolates, that were originally found to be non-binders, isolated from healthy women (10 strains) and from women with bacterial vaginosis (five strains), also showed increased binding of ¹²⁵I-fibronectin at lower pH values. The binding of fibronectin at these low pH values proved to be only partially specific, because only 40–45 % of the radiolabelled fibronectin binding could be inhibited by 100 µg of unlabelled fibronectin or by its 29-kDa fragment. The increased non-specific ¹²⁵I-fibronectin binding at pH 4.0 could be due to non-specific charge interactions taking place at a pH value below the iso-electric point of fibronectin. However, a substantial part of the binding is still specific.

Ultrasound treatment abolished binding to fibronectin, presumably by removing surface structures involved in adherence. After ultrasound treatment the supernate did not itself inhibit binding, which suggests removal was associated with configurational changes.

A great number of pathogenic bacteria belonging to different species have recently been studied in respect of their ability to bind to fibronectin.^{15, 16, 18–20} Their different binding capacities were considered to be an important additional pathogenicity factor for all species studied. However, little is known about the fibronectin binding capacities of the members of the normal bacterial flora and about the possible role of these interactions in the stability of the normal flora on the mucosal surfaces.

Despite the extensive research of the last decades, the vaginal ecosystem is still poorly understood. Different studies show great qualitative and quantitative variations of the occurrence of bacterial species in the vaginal fluid of healthy women. The factors regulating vaginal pH and the role of pH in regulating the vaginal flora are not well defined. We observed markedly increased fibronectin binding with all lactobacilli tested at low pH values. It appears probable that the increased binding of fibronectin by vaginal lactobacilli at these low pH values, which are normal for the vaginal fluid of healthy women in the fertile period of life, plays an additional role in the maintenance of the ecological balance of the vagina.

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