Group B Streptococcus induces tyrosine phosphorylation of annexin V and glutathione S-transferase in human umbilical vein endothelial cells

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Abstract. Group B Streptococcus (GBS), a human pathogen that causes infection and invasive diseases in newborns, pregnant women and immunocompromised adults, has been shown to invade human umbilical vein endothelial cells (HUVECs). The objective of this study was to investigate the molecular mechanisms underlying GBS-HUVEC interaction, focusing specifically on the responsiveness of host protein tyrosine kinase (PTK). We found that GBS serotypes III and V induced actin reorganization and formation of stress fibers into HUVECs. Since rearrangements of the actin cytoskeleton into eukaryotic cells are usually associated with the activation of PTK, we decided to follow the expression of this class of kinases in the course of the interaction. Unexpectedly, treatment of HUVECs with genistein greatly increased both cytoadherence and intracellular viability, for all GBS strains studied. GBS increased tyrosine phosphorylation of two proteins with an apparent molecular mass of 35 and 23 kDa in HUVECs as demonstrated by Western blot analysis with anti-phosphotyrosine antibodies. Mass spectra analysis identified these proteins as annexin V and glutathione S-transferase. Studies are in progress to identify the role of these two proteins on GBS-HUVEC interaction.

Introduction

Streptococcus agalactiae or group B Streptococcus (GBS), a commensal of human intestinal and urogenital flora (1), is a relevant cause of invasive diseases in newborns, pregnant women and immunocompromised patients (2). The clinical scenario concerning invasive diseases caused by GBS underlies the ability of this bacterium to evade host defenses as well as to adhere, bypass and/or injure endothelial cells to gain access to the blood stream (3). Previous studies demonstrated that the GBS-endothelial cell interaction process is a phenomenon of prime importance in the etiology of diseases caused by GBS (4), but an understanding of these events at the molecular level is only in the early stages of characterization.

Pathogenic bacteria use different strategies to invade cultured mammalian cells (5). Human pathogens are able to co-opt non-phagocytic cells by a mechanism in which phosphorylation of tyrosine-rich proteins of host cells is involved (6,7). Protein phosphorylation occurs concomitantly with a drastic rearrangement of actin filaments, which in turn, guide the bacteria during cell invasion (8). Upon GBS binding to host cell receptors, recruitment of host-cell actin to the site of bacteria entry was observed (9).

In a previous study, we observed that phosphorylation of protein kinase C (PKC) in human endothelial cells induced the formation of actin stress fibers and the increase in intracellular viability of GBS-III microorganisms. Conversely, inhibition of protein kinase A (PKA) activity improved the surveillance of GBS type V inside endothelial cells (10). In order to better understand the GBS disease process, our goal in this investigation was to explore the role of the protein tyrosine kinase (PTK) signaling pathway during endothelial cell interaction with GBS.

Materials and methods

Bacteria. GBS serotypes III (80340-vagina carrier and 90356-cerebrospinal fluid [CSF] clinical isolate) and V (88641-vagina carrier and 90186-blood clinical isolate) partially investigated for adhesive properties (10,11) were used in this study. The strains obtained from Culture Collection of the Laboratório de Cocos Patogênicos, Departamento de Microbiologia Médica, Instituto de Microbiologia Professor Paulo de
Góes, Universidade Federal do Rio de Janeiro, Brazil, were identified as group B and serotyped as described previously (12). The microorganism was stored after lyophilization and recovered in Brain Heart Infusion broth (BHI; Oxoid). GBS isolates were cultured on Blood Agar base (Oxoid) plates containing 5% sheep defibrinated blood (BAB/blood) for binding assays.

**Endothelial cell culture.** Human umbilical vein endothelial cells (HUVECs) were obtained by treatment of umbilical veins with a 0.1% collagenase IV (Sigma) solution as previously described (13). Primary cells were seeded onto 25 cm² bottles coated with porcine skin gelatin, and grown in 199 medium (M199)/HEPES (Sigma) supplemented with antibiotics, 100 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 2.5 μg ml⁻¹ anphoteracin-B, 2 mM glutamine, and 20% fetal calf serum at 37°C in a humidified 5% CO₂ atmosphere until they reached confluence. Cells were cultured in 24-plastic wells and only collected after the first or second passages were assayed. Confluent cultures were treated with 0.25% trypsin/0.2% EDTA solution made in 0.01 M phosphate buffered saline (PBS), rinsed in serum-depleted culture medium, and used for experiments.

**Bacterial binding and intracellular viability assays.** Confluent cultures of HUVECs were allowed to interact with GBS (5x10⁷ CFU) treated with 100 μM genistein and 0.01% DMSO (Calbiochem) for 1 h at 37°C in 5% CO₂. For the bacterial binding assays, infected monolayers were rinsed three times with M199, and then lysed in a solution of 0.5 ml of 25 mM Tris, 5 mM EDTA, 150 mM NaCl plus 1% Igepal. The viability of total bacteria (intracellular plus surface adherent) was estimated by plating endothelial lysates and counting the resulting colonies that appeared in BAB plates containing 5% sheep defibrinated blood. To measure bacterial internalization, the infected monolayers were incubated for 1 h, rinsed three times with M199, and then lysed in a solution of 0.5 ml of 25 mM Tris, 5 mM EDTA, 150 mM NaCl plus 1% Igepal. The viability of total bacteria (intracellular plus surface adherent) was determined as outlined above. The adherence rates were determined as follows [CFU of total cell-associated (intracellular viable plus surface adherent) GBS-CFU intracellular GBS] (10).

**Immunoblotting.** HUVEC monolayers infected with GBS (5x10⁷ CFU) for 5 min at 37°C were rinsed with PBS containing 0.4 mM Na₂VO₄ and 1 mM NaF. Next, the infected cells were scraped and incubated for 30 min in a lysis solution (0.4 mM Na₂VO₄, 1 mM NaF, 1% Triton X-100, 100 μM of PMSF, 40 μM of leupeptin and 2 mM EDTA) in 50 mM Tris-HCl (pH 7.6). The extracts were quantified (Bradford), and 15 μg protein were separated by electrophoresis in 12% SDS-PAGE. The resulting bands were stained or not with Coomassie Brilliant Blue G-250 (14). Following SDS-PAGE, the resulting bands were transferred to a nitrocellulose membrane (Amersham Bioscience) by electroblotting. Phosphotyrosine-containing proteins were revealed with the 4G10 anti-phosphotyrosine monoclonal antibody (Molecular Probes), followed by second antibody peroxidase-conjugated. The bound complexes were detected using the enhancement chemiluminescence detection kit (Amersham Bioscience). The molecular mass of the tyrosine-phosphorylated proteins was determined with a Benchmark™ Pre-Stained Protein Ladder (Invitrogen). The bands found in autoradiograms were quantified by scanning densitometry, and the resulting absorbance curves were integrated by using the Scion Image Master. Densitometric analyses were performed on gels with different exposure times, and those giving linear absorbance curves were used to obtain a semi-quantitative assessment.

**MALDI-TOF/TOF analysis and peptide sequencing.** Cell extract proteins, which were separated by SDS-PAGE (1-D electrophoresis), were stained or not with Coomassie Brilliant Blue G-250, and the unstained protein bands were imbibed as detailed above. The SDS-PAGE spots, corresponding to the resulting immunoblotted 25 and 23 kDa bands, were manually excised from 1-D gels and used to extract the tryptic peptides (15). Aliquots (0.5 μl) were eluted from a C₁₈ ZipTip, mixed with α-cyano-4-hydroxycinnamic acid (10 mg ml⁻¹) in acetonitrile/water (50%) containing trifluoroacetic acid (0.1%) and then applied directly onto a target. The tryptic peptide masses were obtained using 4700 Proteomics Analyzer (Applied Biosystems) in an automated mode. Initially, a MALDI MS spectrum was acquired from each spot (1,000 shots/spectrum), and peaks with a signal-to-noise ratio >15 in each spectrum were automatically selected for MS/MS analysis (7,500 shots/
spectrum). Collision energy of 1 keV was used with air as collision gas. Peak lists from all MS/MS spectra were submitted to a database search using an in-house copy of MASCOT, version 3.1 (Matrix Science Inc., Boston, MA). The following criteria were used for all database searches. A minimum signal-to-noise ratio threshold of 5-10; mass values in the range 0-60 Da and masses within 20 Da of the precursor ion mass were excluded; and a maximum of 60 peaks per spectrum were included as product ions. The mass tolerance was ±75 ppm for MS data, ±200 ppm for MS/MS precursor ions, and ±250 ppm for MS/MS product ions. The sample was searched against the NCBInr database (October 4, 2007). The Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB) was used to analyze protein sequences.

**Statistical analysis.** The Student's t-test was used throughout, and p<0.01 was considered significant.

**Results**

**GBS induces actin rearrangement in HUVECs.** HUVECs were treated or not with cytochalasin D prior to their interaction with each of the microorganisms of the assayed GBS strains. The GBS cytoadherence rates to cytochalasin D-treated HUVECs decreased in the order of 50 and 86% for 80340- and 88641-vagina GBS strains, respectively. Nonetheless, increases in cytoadherence of 25% (90356-CSF) and 75% (90186-blood) were detected when GBS microorganisms were assayed (Fig. 1A). After 1 h of HUVECs-GBS interaction we observed an intense actin rearrangement in the host cells. Most HUVECs under interaction with GBS exhibited actin stress fibers, and also a cortical distribution one (Fig. 1D and E) which both in turn, were not observed in non-infected HUVECs (Fig. 1C).

**Cytoadherence and intracellular viability of GBS to genistein-treated HUVECs.** Both cytoadherence and intracellular viability of GBS microorganisms were greatly affected when genistein-treated HUVECs were assayed. As seen in Fig. 2A, previous treatment of HUVECs with genistein resulted in high increases in GBS cytoadherence. The following rates concerning increases in bacterial cytoadherence were detected, four-fold for both 80340-vagina (P<0.001) and 90356-CSF (P<0.0001) microorganisms; one-fold for 88641-vagina (P<0.01) and ~six-fold for the 90186-blood ones (P<0.0001). As revealed by ultrastructural observations, after 1 h of GBS-HUVEC interaction the latter presented GBS associated outside (Fig. 2C) and inside (Fig. 2D) HUVECs. It is important to note that previous treatment of HUVECs with genistein changed, to a great extent, the number of viable GBS recovered inside host cells (Fig. 2B), except for the 90186-blood one that exhibited an intracellular surveillance higher than controls. Compared to controls, the following increased rates related to GBS interaction with genistein-treated HUVECs were obtained: 2-fold for 90356-CSF microorganisms (P<0.01) and ~1-fold for the 88641-vagina ones (P<0.01). The non-invasive 80340-vagina microorganisms were viable inside genistein-treated HUVECs (1.2x10³ CFU ml⁻¹; P<0.0001).

**The association of GBS to HUVECs induces tyrosine phosphorylation in host cell proteins.** GBS microorganisms of both serotypes III (80340-vagina and 90356-CSF) and V (88641-vagina and 90186-blood) were allowed or not to
interact with monolayers formed by HUVECs. The host cell extracts were electrophorezed, blotted onto nitrocellulose sheets, and were finally let to sequentially react with an anti-phosphotyrosine monoclonal and a peroxidase conjugated-secondary antibody. We observed a remarkable peroxidase labelling in both 35- and 23-kDa bands of control HUVECs. However, such immunolabeling of HUVEC proteins was significantly altered after the interaction of GBS with HUVECs (Fig. 3A). The related densitometric measurements revealed that all GBS strains induced increases in phosphorylation levels of these two protein bands (Fig. 3B).

Peptides sequencing. An initial screening using 1D electrophoresis was performed in order to identify the two protein bands phosphorylated to a great extent in HUVECs under interaction with GBS. To identify the 35- and 23-kDa antigen, the corresponding protein bands were excized from the Coomassie blue-stained gel pieces following an in-gel tryptic digestion. The tryptic peptides were further analyzed by mass spectrometry (MALDI-TOF-TOF) and 13 peptides were identified for the 35-kDa band (Table I). A Matrix Science Mascot search and Expasy Blast form analysis revealed homology of these peptides with the Annexin V of *Homo sapiens* (P08758). For the 23-kDa band 2 peptides were identified (Table II). The same Matrix Science Mascot search and Expasy Blast form analysis identified these peptides as being part of the glutathione S-transferase of *Homo sapiens* (P09211).

Discussion

The ability of GBS to survive in host compartments requires the coordinate expression and regulation of multiple pathogenic factors (17). Many important virulence factors contribute to...
GBS virulence, and are being analyzed, but the cellular and molecular mechanisms related to cellular injury and access to central nervous system remains unknown. The elucidation of transduction signals involved in pathogenesis of GBS may contribute to improve the treatment of GBS sepsis.

While it was well established that GBS is able to interact with receptors on the surface of host cells and invade a variety of host cell types, including epithelial and endothelial cells (10,18), there is still few information on their physiologic substrates, regulation and control of signaling networks (19,20). In order to investigate the molecular mechanisms underlying GBS-host cell interactions, we focused on the signal transduction pathways triggered by GBS to human umbilical vein endothelial cells (HUVECs). We designed experiments concerning the involvement of each PTK expression and the organization of actin filaments of HUVECs during their interaction with GBS serotypes III (80340-vagina and 90356-CSF) and V (88641-vagina and 90186-blood).

Most of the invasive pathogens entered into host cells by exploitation of specific mechanisms such as phagocytosis or receptor-mediated endocytosis (21). Similar to most invasive bacteria, the uptake of GBS by HUVECs is a process highly sensitive to cytochalasin treatment. A complete inhibition of intracellular viability of GBS was observed when cytochalasin

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Table I. Peptide sequences identified from the mass spectrometry analysis of the 35-kDa protein.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Experimental [M+H] +</th>
<th>Calculated [M+H] +</th>
<th>Peptide ion score</th>
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<tbody>
<tr>
<td>GTVDTPGFDER</td>
<td>1339.61</td>
<td>1339.60</td>
<td>92</td>
</tr>
<tr>
<td>GLGDEESILTLTSR</td>
<td>1703.89</td>
<td>1703.89</td>
<td>75</td>
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<tr>
<td>SNAQREISAAFK</td>
<td>1448.73</td>
<td>1448.74</td>
<td>88</td>
</tr>
<tr>
<td>LYTAYELKHALK</td>
<td>1013.51</td>
<td>1013.51</td>
<td>67</td>
</tr>
<tr>
<td>VLTEHASR</td>
<td>1000.60</td>
<td>1000.59</td>
<td>42</td>
</tr>
<tr>
<td>QYYSYEGSSLEDVVGDTSGYQR</td>
<td>2887.23</td>
<td>2887.23</td>
<td>149</td>
</tr>
<tr>
<td>WGDEDEEPITIFGTR</td>
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<td>1798.89</td>
<td>116</td>
</tr>
<tr>
<td>VDFKYMISGQIEETIDR</td>
<td>2291.13</td>
<td>2291.11</td>
<td>132</td>
</tr>
<tr>
<td>ETSGNLEQLLAVVK</td>
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<td>SIPAYLAETLYIAMK</td>
<td>1748.87</td>
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<td>111</td>
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<td>GAGTDHALLIR</td>
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<td>87</td>
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<tr>
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<td>NFATSLSYMK</td>
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Peptides identified by MS/MS are underlined in the protein sequence (57% coverage).

Table II. Peptide sequences identified from the mass spectrometry analysis of the 23-kDa protein.

<table>
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<tr>
<th>Peptide sequence</th>
<th>Experimental [M+H] +</th>
<th>Calculated [M+H] +</th>
<th>Peptide ions score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPYTVVYFPVR</td>
<td>1336.70</td>
<td>1336.72</td>
<td>53</td>
</tr>
<tr>
<td>FQDGDLTLQNSNTILR</td>
<td>1882.93</td>
<td>1882.94</td>
<td>120</td>
</tr>
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</table>

Peptides identified by MS/MS are underlined in the protein sequence (12% coverage).
D-treated HUVECs were assayed, which demonstrate that the integrity of the actin cytoskeleton is crucial to the uptake of GBS, and this is in agreement with other studies (3.9). Our findings also related to the partial inhibition of cytoadherence of isolates from asymptomatic carriers (80340- and 88641-vagina) similar to that verified by other pathogen. The reduction in both cytoadherence and uptake of *Pseudomonas aeruginosa* caused by previous treatment of A549 respiratory cells with cytochalasin D was reported (22). In this work, an increase was also noted on adherence of clinical isolates (90356-CSF and 90186-blood) to HUVECs. Studies carried out with *Campylobacter jejune* demonstrated that cytochalasin D resulted in a dose-dependent increase in binding and a dose-dependent decrease internalization of two clinical isolates to human embryonic intestine-INT 407 cells (23). In addition, cytochalasin-mediated effects were both strain- and cell line-dependent for the *Campylobacter jejune* and *Citrobacter freundii* strain, being that the interaction between a bacterial ligand and a mammalian cell surface receptor is a sine-qua-non condition to trigger a microtubule-dependent plasma membrane invagination event that leads to bacterial internalization (24).

The HUVECs-GBS interaction induced important alterations in the organization of microfilaments of the host cells. As soon as GBS begins to establish physical contact with the host cell surface, we observed the formation of stress fibers in HUVECs. Such morphological alterations on the cytoskeleton in *vitro* alters endothelial permeability, exposing the subendothelial matrix, which is explored by GBS, as already described for other pathogens (25). It is a point that deserves to be further investigated.

PTK is a set of ‘molecular switches’ directly implicated in multiple facets of cytoskeleton regulation. Although one or more members of this family of phosphoproteins are involved in cell invasion by bacteria, the data published on the role played by PTK in signaling cascades triggered by adhesion of bacteria to cultured mammalian cells are conflicting (26,27). In nonphagocytic cells, for example, activation of PTK triggers an intracellular signaling cascade related to uptake of bacterial pathogens including *Yersinia* sp (28), *Listeria monocytogenes* (26), and *Campylobacter sp* (29). It was also demonstrated that activation of PTK favors the microbicidal activity of phagocytic cells (27). Curiously, the data here show that previous treatment of HUVECs with genistein increased GBS cytoadherence and uptake by HUVECs by increasing 48 and 150% respectively. Such an effect is almost the same as reported for *Coxiella burnetii* (27), but it contrasts those reported for invasion of both Hep-2 cells (30) and HBMEC (31) by GBS. Since we found a high amount of GBS adhered to genistein-treated HUVECs, it is possible that most bound bacteria entered the HUVECs. Also important is that the host cell response varies with the cell type and tissue-specific location; endothelial cells from vein and artery have a distinct response to a common systemic signal (32). Altogether, these data led us to infer that the interaction of GBS with cultured mammalian cells is a multi-factorial phenomenon, where bacterial adhesions consort to promote and improve bacterial cytoadherence which in turn triggers signaling cascades in host cells that improve invasion and bacterial survival inside host cells.

Since most surface molecules involved in signaling transduction pathways are transmembrane glycoproteins presenting intrinsic kinase activities and connect to actin filaments (33), it is expected that the cytoadhesion performed by GBS triggers kinase activation in HUVECs. We found that all strains analyzed induced after GBS-HUVECs interaction an over-expression of two positive labeled phosphotyrosine protein bands with an apparent MW of 35 and 23 kDa. Our results clearly demonstrate that the 35- and 23-kDa bands were specifically phosphorylated in response of HUVECs to GBS since these phosphoproteins were not detected in cell extracts of GBS that were exposed to bare plastic in the absence of endothelial cells (data not shown).

A comparative analysis carried out between the peptides related to the 35- and 23-kDa protein bands with those available in data bases, resulted in high homologies to glutathione S-transferase and annexin V, respectively. Glutathione S-transferases (GST) are ubiquitous proteins whose primary function is related to detoxification of endo- and exogenous toxic molecules, thus protecting cells from oxidative stress (34,35). Previous studies demonstrated that GBS is capable of triggering oxidative burst in mammalian cells (11,36). The increase of the expression of GST in HUVECs under interaction with GBS might represent a protective mechanism displayed by endothelial cells to protect them against oxidative molecules generated or activated during such cell-bacterium interaction, but more experiments are necessary.

Annexin V belongs to a family of at least 13 structurally related mammalian proteins which bind to anionic phospholipids in a Ca++-dependent manner (37). Annexins are implicated in regulation of membrane traffic during exo- and endocytosis, mediation of cytoskeletal-membrane interactions, mitogenic signal transduction, transmembrane ion channel activity, anti-inflammatory properties, and both inhibition of blood coagulation and phospholipase A2 (38). Annexin V is a specific ligand for phosphatidylycerine (PS). Exposure of PS in turn, is one of the main ‘eat me’ signals for phagocytosis of both apoptotic and necrotic cells (39). Therefore, it is possible that in vivo the interaction between GBS and endothelial cells triggers apoptosis of target cells, which might facilitate the hematogenic dissemination of the bacterium, and endothelial cells become susceptible to phagocytosis, but more experiments are necessary.

Collectively, the data indicate that the identification of signaling pathways that modulate both uptake and survival of GBS into endothelial cells brings to light important data concerning the development of new therapeutic strategies to prevent vascular invasion by GBS. The involvement of annexin V and GST in the course of GBS-HUVEC interaction is the subject of ongoing investigation.

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