

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

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Received February 18, 2009; Accepted April 29, 2009

DOI: 10.3892/ijmm_00000245

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

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Key words: Group B *Streptococcus*, GBS-endothelial cell interaction, protein tyrosine kinase, annexin V, glutathione S-transferase

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 desfibrinated 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 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 2.5 µg ml⁻¹ anphotericin-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 0.025% trypsin/0.2% EDTA solution made in 0.01 M phosphate buffered 0.15 M NaCl at pH 7.2 (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 or not with 100 nM cytochalasin D (Sigma) for 20 min, or 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 desfibrinated blood. To measure bacterial internalization, the infected monolayers were incubated for 1 h, rinsed three times with M199 medium, and incubated for an additional 2 h in M199 containing bactericidal amounts of both gentamicin (100 µg ml⁻¹) and penicillin G (5 µg ml⁻¹). The number of internalized bacteria 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).

Immunofluorescence. HUVECs cultured onto glass coverslips were allowed to interact or not with GBS for 1 h at 37°C. The cells were washed with PBS, fixed in 3.7% formaldehyde for 10 min at room temperature and washed again with PBS-BSA (10 min each). Cells were permeabilized with 0.1% Triton X-100 in PBS for 6 min, washed, and stained with 0.1 µg ml⁻¹ fluorescein isothiocyanate-phalloidin for 1 h. Cells were reacted with 0.5 µg ml⁻¹ 4'-6-diamidino-2-phenylindole (DAPI) for 30 min. Fluorescent image capturing was performed with a Nikon Coolpix 995 digital camera coupled to a Nikon Eclipse E400 epifluorescence microscope, using the highest magnification (x1,000).

Scanning electron microscopy. For ultrastructural analysis of the GBS-HUVECs interaction, conventional scanning electron

(SEM) and field emission scanning electron (FESEM) microscopes were used. HUVECs monolayers were infected with GBS for 1 h, washed with PBS and incubated overnight at 4°C in a solution of 3% paraformaldehyde plus 2.5% glutaraldehyde made in 0.1 M cacodylate buffer. The resulting samples were washed and post-fixed in a solution of 1% OsO₄ plus 8 mM potassium ferrocyanide and 10 mM CaCl₂ in 0.1 M cacodylate buffer. After exhaustive washing with PBS, both GBS infected and non-infected cells were dehydrated in a graded series of ethanol, and the surface of some infected monolayers were scraped with scotch tape in order to expose the inner organization of HUVECs. All cells were dried to a critical point with CO₂, and coated with a thin gold layer. The gold-coated samples were then observed in a Zeiss scanning electron microscope and a JEOL field emission scanner, operating at 15 and 10 kV, respectively.

Immunoblotting. HUVEC monolayers infected with GBS (5x10⁷ CFU) for 5 min at 37°C were rinsed with PBS containing 0.4 mM Na₃VO₄ and 1 mM NaF. Next, the infected cells were scraped and incubated for 30 min in a lysis solution (0.4 mM Na₃VO₄, 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 immunoblotted as detailed above. The SDS-PAGE spots, corresponding to the resulting immunolabelled 35 and 23 kDa bands, were manually excised from 1-D electrophoresis as already described (15) and used to extract the tryptic peptides (16). 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/

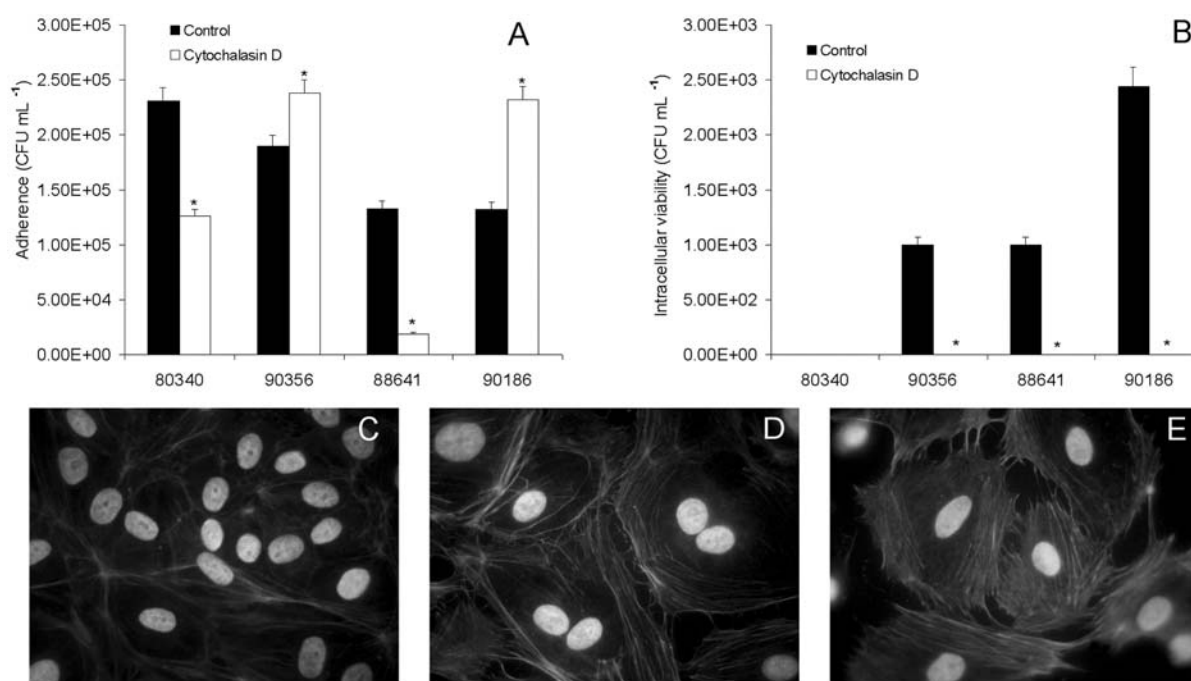


Figure 1. Effect of previous treatment of HUVECs with cytochalasin D in both GBS-HUVECs interaction (A and B) and organization of actin cytoskeleton (C-E). Both GBS cytoadhesion (A) and uptake (B) by HUVECs were altered in cytochalasin D-treated HUVECs (* $p < 0.01$). Reactivity of actin filaments to FITC-phalloidin in non-infected (C) and HUVECs infected with 90356-CSF (D) and 90186-blood (E) GBS microorganisms. Cell nucleus is stained with DAPI. For details see Materials and methods. Magnifications C, D and E, $\times 1,000$.

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 NCBI 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). However, no viability was found in microorganisms of strains which were recovered from lysed cytochalasin D-treated HUVECs (Fig. 1B). 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 (1.2×10^3 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

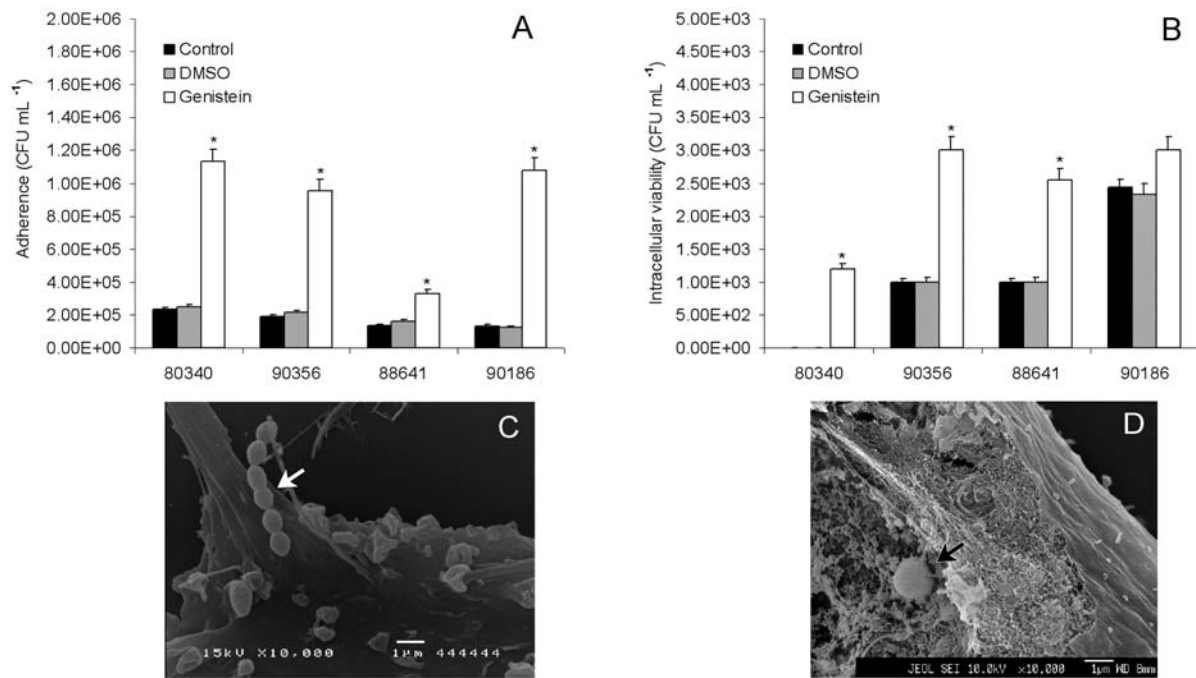


Figure 2. Effect of previous treatment of HUVECs with genistein in the GBS-HUVECs interaction. Monolayers formed by HUVECs were incubated or not with 100 μ M genistein solubilized in 0.01% DMSO prior to their interaction with GBS. Compared to controls, GBS adhered to a great extent to the surface of genistein-treated HUVECs (A and C). GBS uptake by genistein-treated HUVECs was also higher than that found in controls, and most bacteria found inside cells (D) presented high viability indexes (B). * $p \leq 0.01$. See Materials and methods for details. Magnifications C and D, $\times 10,000$.

interact with monolayers formed by HUVECs. The host cell extracts were electrophoresed, 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 immunolabelling 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

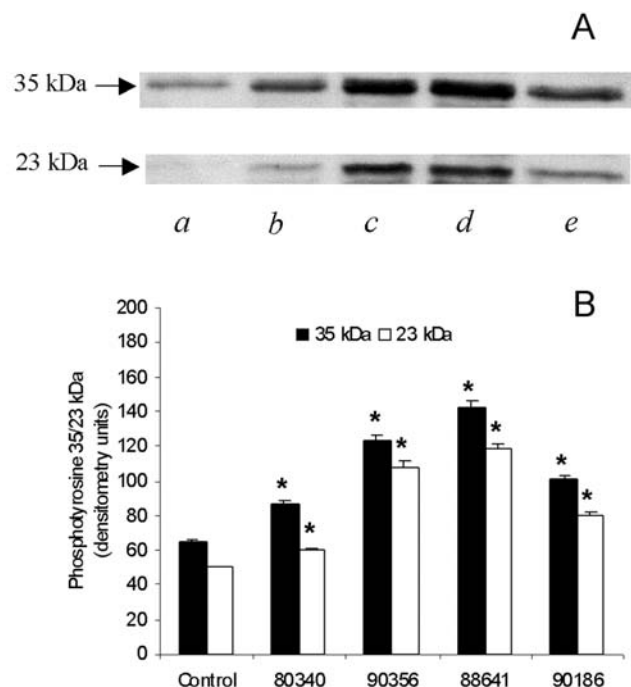


Figure 3. Tyrosine phosphorylation of HUVECs proteins. Whole cell extracts of HUVECs monolayers which interacted or not with GBS (bacterium-cell ratio of 100:1) were processed for immunoblotting as detailed in Materials and methods. Reactivity of HUVECs proteins collected from cells before (a) and after (b-e) GBS-HUVECs interaction was sequentially reacted with an anti-phosphotyrosine monoclonal (4G10 clone) and a peroxidase-conjugated secondary antibody. The reactivity of the antibodies to cell extracts collected from HUVECs before (a) and after interaction with 80340-vagina (b), 90356-CSF (c), 88641-vagina (d), and 90186-blood (e) GBS microorganisms resulted in the appearance of two remarkable protein bands of 35 and 23 kDa (A). The levels of tyrosine phosphorylation found in both 35- and 23-kDa protein bands were assessed by densitometry (B). Results are expressed in absorbance units which in turn, represent the mean values \pm SE of two distinct experiments.

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

1 AQVLRGTVTD FPGFDERADA ETLRKAMKGL GTDEESILTL LTSR SNAQRQ 51 EISAAFKTLF GRDLLDDLKS ELTGKFQKLI VALMKPSRLY DAYELKHALK 101 GAGTNEKVLT EIASRTPEE LRAIKQVYEE EYGSSLEDDV VGDTSGYYQR 151 MLVVLLQANR DPDAGIDEAQ VEQDAQALFQ AGELKWGTDE EKFITIFGTR 201 SVSHLRKVFD KYMTISGFQI EETIDR ETSG NLEQLLAVV KSIRSIPAYL 251 AETLYYAMK GAGTDDHTLIR VMVSRSEIDL FNIRKEFRKN FATSLYSMIK 301 GDTSGDYKKA LLLLCGEDD			
Peptide sequence	Experimental [M+H] ⁺	Calculated [M+H] ⁺	Peptide ion score
GTVTDFPGFDER	1339.61	1339.60	92
GLGTDEESILTLLTSR	1703.89	1703.89	75
SNAQRQEISA AFK	1448.73	1448.74	88
LYDAYELKHALK	1013.51	1013.51	67
VLTEI IASR	1000.60	1000.59	42
QVYEE EYGS SLEDDVVGDTSGYYQR	2887.23	2887.23	149
WGTDEEK FI TIFGTR	1798.88	1798.89	116
VFDKYMTISG FQI EETIDR	2291.13	2291.11	132
ETSGNLEQ LL AVVK	1612.91	1612.90	118
SIPAYLAETLYYAMK	1748.87	1748.87	111
GAGTDDHTLIR	1154.58	1154.57	87
SEIDLFNIRK	1233.68	1233.67	45
NFATSLYSMIK	1273.63	1273.64	48

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.

1 MPPYTVVYFP VRGRCAALRM LLADQGQSWK EGVVTVDTQC GSLKASCLY 51 GELPKFQDGD LTLYQSNTIL RHLGRTLGLY GKDQQEAALV DMVNDGVEDL 101 RCKYISLIYT NYEAGKDDYV KALPGQLKPF ETLLSQNQGG KTFIVGDQIS 151 FADYNLLDLL LIHEVLAPGC LDAFPLLSAY VGRLSARPKL KAFVASPEYV 201 NLPINGNGKQ			
Peptide sequence	Experimental [M+H] ⁺	Calculated [M+H] ⁺	Peptide ions score
PPYTVVYFPVR	1336.70	1336.72	53
FQDGD LTLYQ SNTILR	1882.93	1882.94	120

Peptides identified by MS/MS are underlined in the protein sequence (12% coverage).

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

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 pathogens. 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 A_2 (38). Annexin V is a specific ligand for phosphatidylserine (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.

Acknowledgements

The authors are deeply grateful to both Ms. Mariléa Temperine and Mr. Jorge Nascimento Cardoso for their expert technical assistance. Thanks to Dr Gilberto Domont for his experimental suggestions, and for access to the mass spectrometry facilities at the Proteomics Network of Rio de Janeiro. This work was supported by the following Brazilian agencies: FAPERJ, CNPq, and FUJB-UFRJ. LMLB is a CNPq research fellow.

References

- Moore MR, Schrag SJ and Schuchat A: Effects of intrapartum antimicrobial prophylaxis for prevention of group-B-*Streptococcal* disease on the incidence and ecology of early-onset neonatal sepsis. *Lancet Infect Dis* 3: 201-213, 2003.
- Hull JR, Tamura GS and Castner DG: Interactions of the *Streptococcal* C5a peptidase with human fibronectin. *Acta Biomater* 4: 504-513, 2008.
- Gibson RL, Lee MK, Soderland C, Chi EY and Rubens CE: Group B *Streptococci* invade endothelial cells: Type III capsular polysaccharide attenuates invasion. *Infect Immun* 61: 478-485, 1993.
- Gibson RL, Soderland C, Henderson WR Jr, Chi EY and Rubens CE: Group B *Streptococci* (GBS) injure lung endothelium in vitro: GBS invasion and GBS-induced eicosanoid production is greater with microvascular than with pulmonary artery cells. *Infect Immun* 63: 271-279, 1995.
- Seveau S, Pizarro-Cerda J and Cossart P: Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion. *Microbes Infect* 9: 1167-1175, 2007.
- Münter S, Way M and Frischknecht F: Signaling during pathogen infection. *Sci STKE* (335): re5, 2006.
- Tegtmeyer N, Zabler D, Schmidt D, Hartig R, Brandt S and Backert S: Importance of EGF receptor, HER2/Neu and Erk1/2 kinase signaling for host cell elongation and scattering induced by the *Helicobacter pylori* CagA protein: antagonistic effects of the vacuolating cytotoxin VacA. *Cell Microbiol* 11: 488-505, 2008.
- Rescher U, Ludwig C, Konietzko V, Kharitonov A and Gerke V: Tyrosine phosphorylation of annexin A2 regulates Rho-mediated actin rearrangement and cell adhesion. *J Cell Sci* 121: 2177-2185, 2008.
- Tyrrell GJ, Kennedy A, Shokoples SE and Sherburne RK: Binding and invasion of HeLa and MRC-5 cells by *Streptococcus agalactiae*. *Microbiol* 148: 3921-3931, 2002.
- Santos GS, Lione VO, Costa e Silva Filho F and Nagao PE: Signal transduction in human endothelial cells induced by their interaction with group B *Streptococci*. *Int J Mol Med* 15: 859-863, 2005.
- Teixeira CF, Azevedo NL, Carvalho TMU, Fuentes J and Nagao PE: Cytochemical study of *Streptococcus agalactiae* and macrophage interaction. *Microsc Res Tech* 54: 254-259, 2001.
- Lancefield RC: A serological differentiation of specific types of bovine hemolytic *Streptococci* (group B). *J Exp Med* 59: 441-458, 1934.
- Jaffe EA, Nachman RL, Becker CG and Minick CR: Culture of human endothelial cells of derivate from umbilical cords veins. Identification on by morphologic and immunologic criteria. *J Clin Invest* 52: 2745-2756, 1973.
- Merril CR: Gel-staining techniques. *Methods Enzymol* 182: 477-488, 1990.
- Burnham CAD, Shokoples SE and Tyrrell GJ: Phosphoglycerate kinase inhibits epithelial cell invasion by group B *Streptococci*. *Microb Pathog* 38: 189-200, 2005.
- Hellman U, Wernstedt C, Góñez J and Heldin CH: Improvement of an 'In-Gel' digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal Biochem* 224: 451-455, 1995.
- Tenenbaum T, Bloier C, Adam R, Reinscheid DJ and Schrotten H: Adherence to and invasion of human brain microvascular endothelial cells are promoted by fibrinogen-binding protein FbsA of *Streptococcus agalactiae*. *Infect Immun* 73: 4404-4409, 2005.
- Tamura GS and Rubens CE: Host-bacterial interactions in the pathogenesis of group B *Streptococcal* infections. *Curr Opin Infect Dis* 7: 317-322, 1994.
- Henneke P, Morath S, Uematsu S, et al: Role of lipoteichoic acid in the phagocyte response to group B *Streptococcus*. *J Immunol* 174: 6449-6455, 2005.
- Lione VO, Santos GS, Hirata R Jr, Mattos-Guaraldi AL and Nagao PE: Involvement of intercellular adhesion molecule-1 and beta1 integrin in the internalization process to human endothelial cells of group B *Streptococcus* clinical isolates. *Int J Mol Med* 15: 153-157, 2005.
- Isberg RR and Tran Van Nhieu G: Binding and internalization of microorganisms by integrin receptors. *Trends Microbiol* 2: 10-14, 1994.
- Leroy-Dudal J, Gagniere H, Cossard E, Carreiras F and Di Martino P: Role of alpha5beta1 integrins and vitronectin in *Pseudomonas aeruginosa* PAK interaction with A549 respiratory cells. *Microbes Infect* 6: 875-881, 2004.
- Monteville MR, Yoon JE and Konkel ME: Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer membrane protein and microfilament reorganization. *Microbiol* 149: 153-165, 2003.
- Oelschlaeger TA, Guerry P and Kopecko DJ: Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc Natl Acad Sci USA* 90: 6884-6888, 1993.
- Figueiredo CC, Deccache PM, Lopes-Bezerra LM and Morandi V: TGF-beta1 induces transendothelial migration of the pathogenic fungus *Sporothrix schenckii* by a paracellular route involving extracellular matrix proteins. *Microbiol* 153: 2910-2921, 2007.
- Ireton K, Payrastra B, Chap H, Ogawa W, Sakave H, Kasuga M and Cossart P: A role for phosphoinositide-3-kinase in bacterial invasion. *Science* 274: 780-782, 1996.
- Meconi S, Capo C, Remacle-Bonnet M, Pommier G, Raoult D and Mege JL: Activation of protein tyrosine kinases by *Coxiella burnetii*: role in actin cytoskeleton reorganization and bacterial phagocytosis. *Infect Immun* 69: 2520-2526, 2001.
- Rosenshine I, Duronio V and Finlay BB: Tyrosine protein kinase inhibitors block invasion-promoted bacterial uptake by epithelial cells. *Infect Immun* 60: 2211-2217, 1992.
- Wooldridge KG and Ketley JM: *Campylobacter*-host cell interactions. *Trends Microbiol* 5: 96-102, 1997.
- Valentin-Weigand P, Jungnitz H, Zock A, Rohde M and Chhatwal GS: Characterization of group B *Streptococcal* invasion in Hep-2 epithelial cells. *FEMS Microbiol Lett* 147: 69-74, 1997.
- Shin S, Paul-Satyaseela M, Lee JS, Romer LH and Kim KS: Focal adhesion kinase is involved in type III group B *Streptococcal* invasion of human brain microvascular endothelial cells. *Microb Pathog* 41: 168-173, 2006.
- Aird WC: The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. *Blood* 101: 3765-3777, 2003.
- Hubbard SR and Till JH: Protein tyrosine kinase structure and function. *Annu Rev Biochem* 69: 373-398, 2000.
- Burmeister C, Luersen K, Heinick A, Hussein A, Domagalski M, Walter RD and Liebau E: Oxidative stress in *Caenorhabditis elegans*: protective effects of the Omega class glutathione transferase (GSTO-1). *FASEB J* 22: 343-354, 2008.
- Torres-Rivera A and Landa A: Glutathione transferases from parasites: A biochemical view. *Acta Trop* 105: 99-112, 2008.
- Leib SL, Kim YS, Chow LL, Sheldon RA and Tauber MG: Reactive oxygen intermediates contribute to necrotic and apoptotic neuronal injury in an infant rat model of bacterial meningitis due to group B *Streptococci*. *J Clin Invest* 98: 2632-2639, 1996.
- Mira JP, Dubois T, Oudinet JP, Lukowski S, Russo-Marie F and Geny B: Inhibition of cytosolic phospholipase A2 by annexin V in differentiated permeabilized HL-60 cells. Evidence of crucial importance of domain I type II Ca²⁺-binding site in the mechanism of inhibition. *J Biol Chem* 272: 10474-10482, 1997.
- Rothhut B: Participation of annexins in protein phosphorylation. *Cell Mol Life Sci* 53: 522-526, 1997.
- Green AM and Steinmetz ND: Monitoring apoptosis in real time. *Cancer J* 8: 82-92, 2002.