Adherence and Invasion of Streptococci to Eukaryotic Cells and Their Role in Disease Pathogenesis

Manfred Rohde and G. Singh Chhatwal

Abstract Streptococcal adhesion, invasion, intracellular trafficking, dissemination, and persistence in eukaryotic cells have a variety of implications in the infection pathogenesis. While cell adhesion establishes the initial host contact, adhering bacteria exploit the host cell for their own benefit. Internalization into the host cell is an essential step for bacterial survival and subsequent dissemination and persistence, thus playing a key role in the course of infection. This chapter summarizes the current knowledge about the diverse mechanisms of streptococcal adhesion to and invasion into different eukaryotic cells and the impact on dissemination and persistence which is reflected by consequences for the pathogenesis of streptococcal infections.

Contents

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1 Introduction

Streptococci play an important role as clinically relevant pathogens worldwide. Streptococci cause approximately 700 million human infections each year, with estimated 500,000 deaths (Carapetis 2005). This clinical importance of streptococci was recognized as early as 1879 by Louis Pasteur when studying puerperal fever causing high mortality rates in pregnant women. Thus, streptococci have been recognized as one of the first microorganisms causing contagious disease. Historically, the classification of streptococci was based on Rebecca Lancefield's scheme grouping streptococcal strains according to the different carbohydrate compositions of the cell wall. Nowadays, M- and T- typing is performed or the 5'-end variability of genes for the M protein, *emm* genes, are used for molecular typing. Streptococci consist of a heterogeneous genus that comprises important human pathogens like *Streptococcus pyogenes,* also referred to as Group A Streptococci (GAS), *Streptococcus dysgalactiae* subsp. *equisimilis* as a member of Group C Streptococci (GCS) and Group G Streptococci (GGS), *S. agalactiae* also known as Group B Streptococci (GBS), as well as *S. pneumoniae* (pneumococci). Today, streptococci are also often organized into six groupings (pyogenic, anginosus, mitis, mutans, salivarius, and bovis) based on their 16S rRNA gene sequence. Members of the pyogenic group, *S. pyogenes* and *S. dysgalactiae subsp. equisimilis*, are often associated with a range of diseases including pharyngitis, tonsillitis, impetigo, mastitis, and resulting sequelae like rheumatic fever or glomerulonephritis (Henningham et al. 2012a). Streptococci are also able to cause recurrent infections such as erysipel and tonsillitis. This phenomenon has been described as the carrier stage of streptococcal infection and it is believed that streptococci have a safe ecological niche within the human body, most properly residing intracellularly in eucaryotic cells, which allow them not only to persist but also to resist antibiotic treatment. Moreover, they can lead to severe invasive infections like bacteraemia, cellulites, and necrotizing fasciitis with high mortality due to streptococcal toxic shock syndrome (STSS). This demonstrates that streptococci are able to infect otherwise sterile deep tissue in the human host. For this purpose streptococci express a highly variable and extensive repertoire of virulence determinants that are differentially regulated and expressed in response to signals from the environment within the human host. In the past 15 years more and more evidence has become available which suggests that the long time considered extracellular pathogenic bacteria of the genus *Streptococcus* adhere to host cells, and subsequently invade these cells for their own benefit, namely escaping antibiotic treatment and the host immune system (for reviews see Cunningham 2000; Nitsche-Schmitz et al. 2007; Nobbs et al. 2009; Courtney et al. 2002). This chapter will mainly focus on Group A streptococci highlighting the repertoire of adhesins and invasins of streptococci, and their impact on the colonization and invasion of the human host the persistence of streptococci within the host.

2 Adhesion of GAS

The initial and essential step of streptococcal infectious colonization of the host depends on adhesion of the pathogen to the host tissue. To allow colonization of the site of infection, streptococci express an arsenal of surface bound components, named adhesins, which establish the contact to the host's extracellular matrix (ECM) or directly to host cells. Since streptococci possess multiple adhesins, they are able to colonize various sites in the human body. Adhesion connects the bacteria firmly to the host cells and allows the pathogen to withstand host cleansing mechanisms like mechanical forces by excretion or peristalsis. Nevertheless, in some instances it might be an advantage for the bacteria to detach from a surface in case the growth conditions become unfavorable. Thus, adhesion can sometimes also be considered as being a dynamic process. Most host surfaces are covered by a protective epithelial or endothelial cell layer which is covered with extracellular matrix proteins like collagen, fibrinogen, laminin, vitronectin, or fibronectin (Cremer et al. 1998; Debelle and Tamburro 1999; Schvartz et al. 1999; Pankov and Yamada 2002; Dempfle and Mosesson 2003). Many streptococcal adhesins function by specifically binding to various compounds of the extracellular matrix. Since streptococci express multiple adhesins, it is most likely that different adhesins are expressed under the different environmental conditions encountered throughout an infection. Once adhesion has been established, streptococci can multiply extracellularly, establish colonization of the infection side, internalize into host cells, traffic intracellularly in the host cell and multiply within the cell, or escape from the host cell, or use the host cell as a Trojan horse for systemic spread of the infection.

2.1 Cell Wall-Anchored Adhesins

Adhesins can be attached in four different ways to the streptococcal surface (i) covalently linked through the C-terminus to the cell wall peptidoglycan through an LPxTz motif, (ii) tethered to the cell membrane through N-terminal modifications with lipid, (iii) retained on the surface by a yet unknown mechanism, or (iv) bound back to the cell surface through noncovalent interactions with cell surface compounds like polysaccharides or other proteins. Most adhesins belong to the family of cell wall-anchored proteins. A prerequisite for those proteins is a functioning membrane-associated transpeptidase, sortase A (Maraffini et al. 2006). Many streptococcal adhesins function by specifically recognizing and binding to the various components found in the ECM of the host. The ECM is the major support of cells and tissue and it is responsible for maintaining strength and elasticity of the body. Thus, it is ubiquitously present and frequently exposed under conditions such as trauma and injury, making the then exposed components an ideal prime target for streptococcal adhesion. In addition to the adhesins, other surface

structures of streptococci like fimbriae, pili, lipoteichoic acid, and polysaccharides may also play an important role during the first steps of adhesion to the host cell.

2.1.1 Hyaluronic Acid Capsule and Lipoteichoic Acid

Many of the *β*-hemolytic streptococci are producing a polysaccharide capsule, consisting of hyaluronic acid (HA), a glycosaminoglycan that is a linear polymer of alternating monosaccharide units of *N*-acetylglucosamine and glucuronic acid. Streptococci show differently expressed HA capsules. While some strains are barely encapsulated when grown ex vivo on agar plates, others produce high amounts of the HA capsule that confers a mucoid appearance to the bacterial colonies that were grown on solid media (Wilson 1959). When compared to the host HA, the streptococcal HA is chemically very similar. The host HA is a widely distributed and abundant component of the host's ECM. The chemical similarity might be the reason for the low immunogenicity of the streptococcal HA capsule in the infected patients. It is assumed that the HA capsule masks the bacteria and therefore provides protection against the host immune system. An epidemiological investigation demonstrated that encapsulated strains can often be isolated from severe invasive infections, whereas isolates from uncomplicated pharyngitis were only rarely of mucoid character. A total of 42 % of isolates from patients with acute rheumatic fever were found to be of the mucoid type (Johnson et al. 1992). In addition, the HA capsule can act as a nonprotein adhesin by binding to the hyaluronic acid receptor CD44 on keratinocytes (Schrager et al. 1998), and the HA capsule serves as a ligand for collagen IV (Dinkla et al. 2003a). These studies highlight that the HA capsule is not only a nonprotein adhesin, but can also be described as a virulence factor associated with invasive infections and their severe sequelae like acute rheumatic heart disease. Studies in the US concluded that outbreaks of acute rheumatic fever were associated with the spread of mainly M-type 18 highly encapsulated strains (Veasy et al. 2004; Kaplan et al. 1989). In addition, the contribution of the HA capsule for pharyngeal colonization was also demonstrated (Wessels et al. 1991). Most noteworthy, mucoid strains seem to help to breach epithelial cell layers for entering underlying sterile tissue in the process of dissemination in the host. Binding of the HA capsule to CD44 leads to cytoskeletal rearrangements in human epithelial cells (Schrager et al. 1998; Cywes et al. 2000) that cause disruption of intracellular junctions and allow the passage into deeper tissue. Streptococci therefore travel paracellularly through an epithelial cell barrier, such as keratinocytes, instead of an intracellular passage after internalization into the epithelial host cells (Cywes and Wessels 2001).

Evidence has been acquired demonstrating that the HA capsule is not always representing a nonprotein adhesin, since it was demonstrated that the HA capsule can also counteract internalization into human epithelial cells and keratinocytes (Jadoun et al. 2002; Darmstadt et al. 2000; Schrager et al. 1996). The observed decreased internalization is accompanied by an increase in tissue damage as observed in a mouse model (Schrager et al. 1996). The increased virulence was

also attributed to an antiphagocytic effect of the HA capsule (Wessels et al. 1991, 1994; Wessels and Bronze 1994). The HA capsule was reported to decrease the association with PMNs, thereby counteracting phagocytosis (Dale et al. 1996).

Lipoteichoic acid (LTA) is composed of chain-like glycerol phosphate polymers that are covalently coupled to glycolipids of the plasma membrane and represent a component of the cell wall. LTA is thought to allow streptococci to come into first contact with the ECM or directly with the host cell. A two-step adhesion mechanism has been postulated. LTA has the role of a first-step adhesive component with low cell type selectivity (Beachey and Ofek 1976; Leon and Panos 1990; Courtney and Hasty 1991; Courtney et al. 1992), whereas the highly abundant extracellular ECM protein fibronectin functions as a ligand or "bridging-molecule" for LTA mediating the initial streptococcal cell adhesion (Simpson and Beachey 1983). For establishing a firm adhesion to host cells, a second step with high avidity and cell specificity is required (Courtney et al. 1992; Hasty et al. 1992). This second step has a crucial influence on the observed tissue tropism of streptococcal infection and the pathway of dissemination in the host. More importantly, interactions of the proposed second step were shown to promote internalization of the bacteria into eukaryotic cells (LaPenta et al. 1994, Greco et al. 1995). The involvement of LTA in adhesion and invasion during streptococcal pathogenesis was recently demonstrated. In a cell culture model consisting of human brain microvascular endothelial cells, Group B streptococci (GBS), the leading pathogen in neonatal meningitis, with an impaired anchoring of LTA in the cell wall due to an inactivated glycosyltransferase gene showed a decreased invasion capability. The mutant strain exhibited less virulence in the meningitis mouse model of infection (Doran et al. 2005). Although LTA promotes adhesion in a nonselective way, LTA adhesion might contribute to the onset of the subsequent infection pathway.

2.1.2 Fimbrious Structures Like Pili

Examination of negatively stained streptococci reveals in some groups filamentous structures like fibrils and pili or fimbriae. Fibrils have been detected especially in oral streptococci with a peritrich or specifically localized structure on the cell wall. These structures have been related to growth and survival of oral streptococci since all fresh isolates express fibrils (Handley et al. 1984, 1987).

For many years, bacteriologists had evidence that Gram-negative bacteria adhere to host cells and pili, also sometimes referred to as fimbriae, serving as the primary colonization factor in the adhesion process. Pili are long hair-like extensions of the cell surface and can best be made visible by negative-staining of the bacteria and by electron microscopic observation. Although pili in Gram-positive bacteria were described first in 1968 in Corynebacteria and in the 1990s in streptococci (Yanagawa et al. 1968; Yanagawa and Honda 1976; Wu and Fives-Taylor 1999), they were more or less neglected in research in the years to follow. Just recently, pili in all three pathogenic streptococcal species causing invasive

disease, *S. pyogenes, S. agalactiae,* and *S. pneumonia*, have been described (Mora et al. 2005; Lauer et al. 2005; Rosini et al. 2006; Barocchi et al. 2006). Since then, pili have come into the research focus and the newly detected pili in streptococci have been considered as most promising candidates for vaccine development against streptococci (Gianfoldoni et al. 2007).

In Gram-negative pathogens, pili consist of multiple subunits which are non-covalently attached to each other and the adhesin molecule is located exclusively at the tip of the pili. There are remarkable differences in the pili assembly in Gram-negative and Gram-positive bacteria. Whereas in Gram-negative bacteria the Sec-dependent secretion and chaperones are involved in the formation of the pili subunits (backbone) and the tip adhesin subunit (the most common assembly way is the chaperone/usher pathway) (Saulino et al. 2000), in Gram-positive bacteria pili subunits are covalently linked to each other and pili are covalently attached to the peptidoglycan. As in Gram-negative bacteria, the Sec-dependent machinery is also involved, but the polymerization of the subunit is controlled by an enzyme with homologies to sortases, called sortase A, which sorts and attaches proteins covalently to the peptidoglycan. For covalent linkage to the peptidoglycan, proteins exhibit a specific motif, the cell wall sorting signal (CWSS), in the C-terminus of the protein. The main characteristic is the LPxTG motif, a sequence that is conserved in all surface proteins anchored by sortase A. The LPxTG motive is followed by a hydrophobic membrane-spanning domain and a positively charged tail which is needed for the sortase A catalyzed anchoring with the peptidoglycan. For anchoring a surface protein to the cell wall, sortase A cleaves the LPxTG motif between threonine (T) and glycine (G), and the threonine residue of the cleaved peptide is covalently linked to the amino group of the cross-bridge within the peptidoglycan (Schneewind et al. 1993; Ton-That et al. 2004; Telford et al. 2006; Scott and Zähner 2006; Mandlik et al. 2007a). During the polymerization process, self-generated intramolecular isopeptide bonds are formed which give additional stabilization to the thin pilus structure and allow to withstand the tensile forces during the first steps in adhesion to the host cells. Remarkably, similar isopeptide bonds have been found in other surface displayed adhesins (Kang et al. 2007). In addition, genes encoding transpeptidases of the sortase C subfamily have been found functioning in polymerization of the subunits (Proft and Baker 2009).

For all streptococci it was found that the genes encoding for pilus proteins are located on pathogenicity islands (PI) and clustered at the same genetic locus in an operon. Sortase genes are in close proximity to the pili genes, implying that they might also belong to the operon. Up to now, nine PIs have been identified in GAS. Remarkably, the genes in GAS occur in the fibronectin binding, collagen binding, T-antigen region of the chromosome (FCT-region). Interestingly, this locus actually encodes the Lancefield T-antigen which has been used for serotype typing (Falugi et al. 2008). Mora et al. (2005) first identified that the T-antigen of serotyping actually represents the shaft of the pilus in a set of four GAS strains of different serotypes. Immune negative-staining with an antibody against the T-antigen revealed staining alongside the entire shaft of the pilus, but no labeling was

detected at the pilus tip. The tip region was only stained by an antibody against the presumed adhesin. Thus, the pilus shaft represents the T-antigen used by R. Lancefield for serotyping. Additionally, a third component was identified in Gram-positive pili which is added at intervals into the shaft region of the pilus, the AP1 (ancillary protein 1) component, representing a collagen binding protein moiety (Podbielski et al. 1999). This observation confirms that pili are involved in the adhesion process especially with components of the ECM. Since pili were detected in streptococci, more evidence has been accumulating that pili are involved in the adhesion process, and in fact pili might actually mediate the first contact with the host cell. Subsequently, it was demonstrated that pili are involved in the adhesion process to a broad range of host epithelial cells including cells from the nasopharynx, tonsils, lung, cervix, and intestine (Abbot et al. 2007; Crotty et al. 2010). Remarkably, for GBS it was found that despite being involved in the adhesion process, pili in GBS are responsible for a paracellular passage through an epithelial cell barrier as well as for promoting invasion into brain microvascular endothelial cells (Maisey et al. 2007; Pezzicoli et al. 2008). Recent pili research has highlighted that pilus protein components of invasive streptococci have amino acid sequences similar to those of the members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which have been shown to interact with the ECM proteins like fibronectin, fibrinogen, collagen, laminin, and vitronectin (Patti and Höök 1994). Pili have also been shown to be responsible for tissue tropism during infections. Besides the long stretched extended pili, the so-called minor pilins have been identified in *Corynebacterium* by mutation analyses of pili. For these minor pilins, it has been demonstrated that they are displayed on the cell surface. Recombinant protein, when coated onto inert latex beads, clearly exhibited binding only to pharyngeal cells, but no binding resulted to cells of lung or laryngeal origin when tested in a cell culturing model (Mandlik et al. 2007b). In addition, the role of minor pilins in streptococcal tissue tropism has also been elucidated. These minor pilins mediate adhesion of GAS to human tonsil epithelium and primary keratinocytes, the prime colonization targets of GAS (Abbott et al. 2007).

From these observations, a putative model of cell adhesion through pili has been deduced (Telford et al. 2006). The first contact with the ECM of the host or directly with the host cell surface is mediated by the extended pili with the presumed adhesin (AC2-protein) on the tips of the pili. This interaction might be reversible to allow streptococci to find the specific tissue (cell tropism). Once the first contact has been established, the Ap1-protein can interact with collagen to attach the bacterium more firmly to the host cell. Due to this interaction, the bacterium comes closer to the host cell surface (in Gram-negative bacteria it has been shown that after the first attachment pili are retracted to get the bacteria closer to the cell surface) and cell surface-anchored adhesins can establish the intimate contact with the host cell and subsequently colonize the cell or tissue. The intimate contact favors three different functions which are needed to progress with the infection: (i) the proximity of the bacterial cell wall with the host cell membrane allows further ligand–receptor interactions, (ii) the close contact favors the

efficient delivery of virulence factors as for example for the type three secretion system, TTSS, in Gram-negative bacteria, and (iii) the intimate adherence between streptococci and host cell membrane is the prerequisite for an successful invasion of the host.

2.1.3 M Proteins

M proteins were the first reported adhesins of GAS (Ellen and Gibbons 1972, 1974) and represent multifunctional virulence factors and adhesins of the streptococcal surface. Although structurally closely related, M proteins represent a heterogeneous group of adhesins with respect to their ligand molecules or target cells (Berkower et al. 1999). Some M proteins exhibit very distinct binding properties, whereas the interaction with glycosaminoglycanes represents a more common adherence mechanism displayed by several M protein serotypes. These interactions are mediated via the conserved C-terminal part of M protein (Frick et al. 2003). M proteins are hypervariable in the N-terminal region of the protein and today more than 200 emm-serotypes can be distinguished which differ in their adhesion capabilities (Beall et al. 1996). M proteins consist of four repeat regions with the A repeats region being hypervariable and the B repeat regions being semivariable. Structural-based studies revealed that M proteins are entirely α-helical coiled-coil dimers and can be detected on the bacterial surface as hair-like projections in ultra-thin sections. The N-terminal region is further stabilized by antiparallel interactions, which is thought to result in bacterial aggregation (Frick et al. 2000; Oemcke et al. 2010). The hypervariable regions of M proteins bind to a wide range of different human proteins including plasminogen, IgA, IgG, factor H, and C4b-binding protein (C4BP) which inhibit complement activation (Andre et al. 2006, McArthur and Walker 2006). The B repeats can bind to fibrinogen, human serum albumin, and IgG. For the ability to bind fibrinogen, it was shown that structural irregularities and instabilities in the coiled-coil structure of M1 protein facilitate binding to fibrinogen (McNamara et al. 2008). In addition, serotype M6 protein can bind to the membrane-bound cofactor protein CD46 on keratinocytes. The C-terminal region of M6 protein as well as the short consensus domains 3 and 4 of CD46 was shown to be crucial for M6/CD46-mediated keratinocyte attachment (Okada et al. 1995; Giannakis et al. 2002). The direct involvement of M proteins in cellular adhesion has been demonstrated for several M protein serotypes. For example, for M1 protein, it was shown that the ECM protein fibronectin is necessary for efficient adhesion to epithelial HeLa cells. As the cellular receptor for fibronectin $\alpha_5\beta_1$ integrins have been identified. An M1 protein mutant showed significant reduced adhesion which could be blocked by specific M1 protein antibodies (Cue et al. 2000, 2001). Similar results were observed for the M24 serotype protein in which the serotype M24 protein mediated adhesion to HEp-2 cells, whereas the M24 protein deficient mutant was unable to bind to HEp-2 cells but was able to adhere to buccal cells demonstrating the cell type specificity (cell tropism) of the M24 protein. The mutant studies also revealed

that another adhesin or adhesins other than M24 protein must be displayed on the bacterial surface (Courtney et al. 1994). Using the cell culture approach, other M proteins have been tested for their involvement in adhesion to epithelial cells. For example, for the M6 protein it was shown that M6 protein does not contribute to the adhesion to buccal and tonsillar epithelial cells. In these studies, again the involvement of fibronectin in the adhesion process was demonstrated (Caparon et al. 1991). The M3 protein serves as an important adhesin for soluble type I and type IV collagen as well as to native collagen matrices in the host. The N-terminal variable region, though specific for the M3 protein, was identified as the collagen binding region. This explains why all other M proteins lack the collagen binding properties of the M3 protein. Besides direct collagen binding, M3 protein is responsible for aggregation of soluble collagen, resulting in large aggregates of streptococci that are involved in the colonization process. Due to this aggregate formation streptococci can better resist phagocytosis and antibiotic treatment (Dinkla et al. 2003b). *S. pyogenes* expresses only one collagen binding protein, Cpa, which was identified in a serotype M49 isolate and was shown to mediate attachment to immobilized type I collagen (Podbielski et al. 1999). Nevertheless, the potential role of collagen binding by serotype M3 protein in the adhesion to host tissue has to be demonstrated. That collagen can play a role in cell adhesion can be deduced from investigations on GGS. In strains of this group, the M-like protein FOG is expressed which has a high affinity for collagen I and allows GGS to bind to human foreskin fibroblasts (Nitsche et al. 2006). Since it is known that two distinct collagen-like proteins, termed SclA/Scl1 and SclB/Scl2, can bind to pharyngeal and fibroblast cells reacting with the α2-domain of $\alpha_2\beta_1$-integrin, and thereby triggering cellular signaling in lung fibroblasts, the conclusion could be draw that GAS employs collagen binding integrins to adhere to host cells (Humtsoe et al. 2005).

Taken together, the role of M protein-mediated adhesion varies obviously with the M-serotype and represents a concerted action of the different ligand binding properties and sites within the M protein. We are just beginning to understand parts of the complex cell adhesion interactions mediated by the M proteins with respect to ligand–receptor binding. Once this process is fully understood, one might gain an insight into the so far mostly unknown interactions in streptococcal cell tropism and the onset of the cell tropism on the development of the disease.

2.1.4 Fibronectin-Binding Proteins

Fibronectin is a large glycoprotein which exists both as a soluble protein in plasma and as a fibrillar polymer in the ECM. Fibronectin is composed as a dimer of two 250 kDa subunits which are C-terminal linked via disulfide bonds. Each subunit exhibits three distinct modules, the type I, II, and III modules. Fibronectin interacts with host cell surface exposed integrins of which the $\alpha_5\beta_1$ integrin is the classical fibronectin-binding integrin. Integrin binding is mediated through the RGD sequence within the fibronectin subunits (Pankov and Yamada 2002). Fibronectin

can be seen as one of the prime targets in streptococcal adhesion and subsequent internalization when exploring the ECM during first steps of adhesion. Fibronectin acts as a bridging molecule, connecting bacterial adhesins with integrins on the surface of eukaryotic cells. All streptococci express fibronectin-binding proteins, but the proteins show differences in the fibronectin-binding capacities. Some serotype strains bind soluble fibronectin with high affinities (in the nanomolar range), whereas other strains can only adhere to immobilized fibronectin for a successful adherence. *S. pyogenes* possesses at least 11 distinct fibronectin-binding adhesins including SfbI/F1, protein F2, serum opacity factor (SOF), FbaA, and several M proteins. Some of these are present in a large number of serotypes, such as SfbI protein or FBP54, whereas others like the M1 or M3 proteins are exclusively expressed by M1 or M3 serotype streptococci (Talay et al. 1992, 1994; Hanski and Caparon 1992; Sela et al. 1993; Natanson et al. 1995; Ozeri et al. 1996; Cue et al. 2000; Kreikemeyer et al. 2004a). The expression of these fibronectin-binding proteins is highly regulated in response to environmental signals. For example, SfbI expression is regulated by a superoxide signal. At high partial pressures of O_2, expression of SfbI is increased while expression of M protein is upregulated in a CO_2-rich environment, which suggests that SfbI protein is expressed at the prime target sites of *S. pyogenes* adhesion, namely in the O_2-rich environment of the respiratory tract and skin, where it is required for host adhesion and colonization. Whereas in deeper infections protection against the immune system becomes more important, the increased partial pressure of CO_2 upregulates M protein (Gibson et al. 1995; Gibson and Caparon 1996; Kreikemeyer et al. 2003, 2004b).

Among all fibronectin-binding adhesins of *S. pyogenes*, SfbI protein and its allelic variant F1 are studied most extensively. Identified in 1992, SfbI/F1 was shown to act as an adhesin on epithelial cells (Talay et al. 1992; Hanski and Caparon 1992). SfbI protein exhibits a modular architecture with an aromatic domain, rich in aromatic amino acids, ARO, at the N-terminus, a proline-rich repeat region (PRR) in the middle of the molecule and a fibronectin-binding repeat region (FnBR) at the C-terminus as major modules (Talay et al. 1994). SfbI protein is very variable in the number of repeats in the PRR and FnBR regions. One study revealed that 34 distinct alleles of SfbI were found in 54 *S. pyogenes* strains as a result of horizontal gene transfer (Towers et al. 2003). The ARO region showed a high degree of sequence variability, whereas deletion or duplication of repeat units in PRR and FnBR resulted in variable numbers of PRR (1–11 repeats) and FnBR (1–5 repeats). Thus, SfbI protein exhibits a high antigenic variation, which might also reflect possible variable functional capabilities.

Binding to fibronectin is mediated by two distinct domains (Sela et al. 1993; Ozeri et al. 1996) and proceeds in the following way: the C-terminal fibronectin-binding repeat region and the adjacent nonrepetitive domain termed spacer 2 or UR synergistically bind to two distinct regions on the fibronectin molecule: the N-terminal fibrin-binding fragment harboring fibronectin F1 modules 1–5, and the gelatine/collagen-binding fragment harboring F1 modules 6–9 and the two F2 modules. Due to this cooperative binding, the RGD region in fibronectin gets exposed and can bind to integrins (Talay et al. 2000).

The molecular interaction between SfbI and fibronectin is based on three-dimensional structural data. Three-dimensional structure data are available describing a bacterial fibronectin-binding fragment of *S. dysgalactiae* bound to its target (Schwarz-Linek et al. 2003, 2004a, b). Since SfbI shows high similarity in respect to structural composition, conclusion can be drawn on the binding mechanism for SfbI and fibronectin. That this can indeed be observed was recently shown by Marjenberg et al. (2011) by applying microcalorimetry to reveal cooperative binding of fibronectin fragments to arrays of binding in SfbI (Marjenberg et al. 2011). The so far existing structural model for fibronectin-binding proteins is comprehensively reviewed by Schwarz-Linek et al. (2004a, b). Briefly, SfbI and fibronectin bind to each other in an antiparallel fashion. The C-terminal FnBR in SfbI recognizes the N-terminal domain of fibronectin with high specificity and high affinity (in the nanomolar range) by forming a novel protein–protein interaction mechanism, termed tandem ß-zipper. According to the tandem ß-zipper model, FnBRs can bind multiple copies of fibronectin. For SfbI from a *S. pyogenes* strain, it was demonstrated that a single SfbI molecule can bind up to five fibronectin molecules. The observed high affinity is considerably important since high-affinity binding is a prerequisite for firm bacterial attachment, because adherent streptococci have to withstand shear forces occurring on the mucosal surfaces or during the internalization process itself.

The C-terminal fibronectin-binding repeat region of SfbI was demonstrated to be sufficient to mediate adherence to epithelial cells (Talay et al. 2000). Several studies revealed that SfbI mediates attachment to epithelial cells of the oral mucosa and the lung, but also to endothelial cells (Rohde et al. 2003). Besides its potential to bind to cell exposed integrins, SfbI has the ability to recruit collagen via prebound fibronectin. This allows *S. pyogenes* to form aggregates and renders the organism capable of colonizing collagen matrices within the body (Dinkla et al. 2003a) as depicted in Fig. 1.

Protein F2 or PFBP are homologous but distinct fibronectin-binding proteins, found in most isolates of *S. pyogenes* lacking the *sfbI*/*prtF1* gene (Jaffe et al. 1996; Rocha and Fischetti 1999; Kreikemeyer et al. 2004a). Protein F2 possesses two binding domains that interact with fibronectin. The most abundant fibronectin-binding protein found in all *S. pyogenes* isolates is FBP54. Although lacking a classical membrane anchor motif of Gram-positive surface proteins, it seems to be localized on the streptococcal surface by a distinct mechanism, thereby acting as an adhesin for buccal epithelial cells but not HEp2 cells (Courtney et al. 1996; Chhatwal 2002). In summary, this data suggests that distinct fibronectin-binding proteins target different cell types, and therefore might contribute to streptococcal cell tropism.

Streptococci can express two other fibronectin-binding proteins, Fba and FbaB. The *fba* gene was found only in five serotypes of *S. pyogenes* including serotype M1 and M49. An Fba mutant showed reduced adhesion to HEp2 cells, suggesting that this protein has adhesive properties. Interestingly, FbaB protein was only found in serotype M3/M18 *S. pyogenes* isolates and appears to be genetically most closely related to protein F2 (Terao et al. 2001, 2002). FbaB protein from serotype M3 *S. pyogenes* exhibits cell tropism since it mediates adhesion only to endothelial and not to epithelial cells (Amelung et al. 2011).

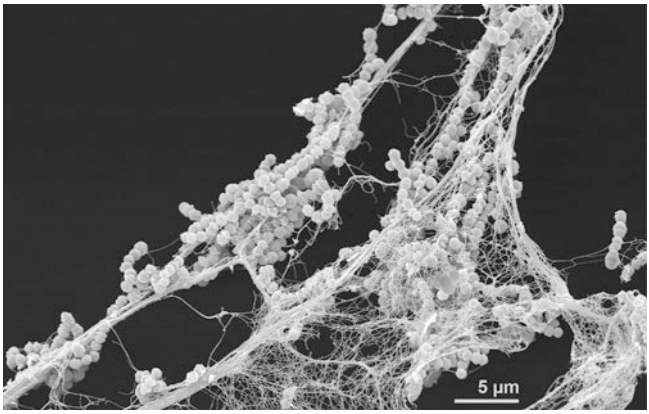

Fig. 1 Field emission scanning electron microscopic (FESEM) image of direct binding of serotype M3-type *Streptococcus pyogenes* to mouse collagen I

Another fibronectin-binding protein exposed on the bacterial surface is Protein H, a member of the M protein family. Protein H binds to the type III modules of fibronectin, unlike the so far described proteins that mainly interact with the type I or type II module containing domains of fibronectin. In addition, Protein H was shown to mediate streptococcal aggregation through a so called AHP sequence that also promoted adhesion to epithelial cells (Frick et al. 1995, 2000). M1 protein, another member of the M protein family, also binds to fibronectin and as in the case with SfbI, $\alpha_5\beta_1$ integrins are the terminal receptor proteins on the cellular surface. M1 specific antibodies efficiently blocked adherence to epithelial HeLa cells. Moreover, an M1-deficient mutant showed reduced adherence indicating that M1 protein acts as an adhesin in serotype M1 *S. pyogenes* strains (Cue et al. 1998, 2000; Dombek et al. 1999).

2.2 Anchorless Adhesins

Most streptococcal surface proteins are attached through the C-termini via an LPxTz motif to the peptidoglycan of the cell wall. In recent years, a few proteins have been identified that bind to the cell surface without the LPxTz motif- the so called anchorless proteins. Noteworthy, these proteins also lack the N-terminal signal sequence. How these proteins are exported from the cytoplasm to the cell surface is not understood and remains an enigma as well as the attachment mechanism to the cell wall. Since the anchorless adhesins do not contain choline-binding repeats and can be removed from the bacterial surface with chaotropic agents, the interaction seems to be of hydrophobic nature, van der Waals forces, or of less-defined charges (Derbise et al. 2004). Anchorless adhesins are structurally

and functionally very diverse and bind to different ligands. Most important is the fact that many anchorless adhesins display an enzymatic function mostly in the cytoplasm of the bacterium, especially in the glycolytic cycle. Triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase, 3-phosphoglycerate mutase, and α-enolase are the last five enzymes in the glycolytic cycle and all have been found to be associated with the cell wall surface. Up to date, from these GAPDH and α-enolase are the best studied examples (Pancholi and Fischetti 1992, 1998; Kinnby et al. 2008). GAPDH can be detected in several species of streptococcus and targets multiple matrix proteins like fibronectin, fibrinogen, plasmin, and plasminogen. It is discussed that GAPDH contributes significantly to the colonization capabilities of GAS. Surprisingly, GAPDH seems also to be involved in interactions with the cytoskeletal proteins actin and myosin and with urokinase plasminogen activator receptor (Pancholi and Fischetti, 1992; Seifert et al. 2003; Jin et al. 2005). The above described anchorless adhesins have another feature in common when they function as a complex in the glycolytic cycle namely generation of ATP. Thus, the anchorless adhesins allow streptococci to produce extracellular ATP on the surface. It can be speculated that streptococci might use this ATP for host modulatory effects, since it is known that ATP is able to bind to $P2X_7$ receptors on immune cells and can inhibit apoptosis. Streptococci seem to have evolved a system to manipulate the host immune cells for the benefit of a better progression of the infection after the first steps of adhesion to the host tissue (Yilmaz et al. 2008).

α-enolase serves as the major plasmin and plasminogen-binding protein of streptococci. After binding plasminogen, it can be converted into plasmin by plasminogen activators. Plasmin represents a very potent serine protease which degrades ECM matrix proteins to allow streptococci to gain access into deeper tissue. It is also discussed if plasmin is involved in facilitating bacterial dissemination through epithelial or endothelial barriers. Other members of anchorless adhesins are GtfG, SpeB, HtrA, Sib35, and FBP54. GtfG, SpeB, and Sib35 are secreted proteins which bind back to the cell wall surface (Hytonen et al. 2001; Kawabat et al. 2002). Other anchorless proteins like ADI, TF, and FBA were found by a post-proteomic approach. For ADI and TF, it was shown that both proteins elicited a protective immune response against intraperitoneal challenge with a serotype M1 isolate (Henningham et al. 2012b).

In summary, anchorless adhesins allow streptococci more flexibility compared to cell wall-linked adhesins since the anchorless proteins can be released and then bound back to the cell wall surface for better exploiting the environment by interacting with host molecules in a certain distance away from the bacterium. The question whether the glycolytic enzymes produce extracellular ATP or not and the function of extracellular ATP in the infection process has to be answered in further studies.

3 Invasion of Streptococci into Eukaryotic Cells

As outlined above, receptor recognition via ECM proteins as bridging molecules for adhesins is essential for a successful adherence to host tissue or cells. During the last two decades, it has become clear that after adhesion a subsequent process is triggered by the firmly attached streptococci, namely internalization into host cells. As mentioned, integrins play an important role in adhesion to the ECM proteins bound by streptococci and the integrin signaling pathways are then exploited by the bacteria to promote their own uptake by an outside–inside signal.

In 1994, LaPenta and colleagues first demonstrated in cell culture infection models that GAS can invade into nonphagocytic human epithelial cells at frequencies equal or greater than the classical intracellular pathogens such as *Listeria* or *Salmonella* (LaPenta et al. 1994). These experiments revealed that the long standing view of streptococci as being only extracellular human pathogenic bacteria has to be changed. These cell culture model studies were followed by the observation that excised tonsils from patients with recurrent tonsillitis contained viable streptococci (Oesterlund and Engstrand 1997; Oesterlund et al. 1997). Furthermore, it was demonstrated that besides GAS, other streptococci also invade nonphagocytic epithelial host cells such as Group B, Group C, and Group G streptococci, *S. suis* as well as *S. pneumoniae* and oral streptococci of the viridans group (Rubens et al. 1992; Talbot et al. 1996; Norton et al. 1999; Molinari and Chhatwal 1999; Haidan et al. 2000; Stinson et al. 2003).

Scanning electron microscopy (SEM) imaging revealed that streptococci use multiple morphological ways of invasion. This became evident when different serotypes with most probably different adhesins were tested for invasion. All invasion mechanism known so far of the classical intracellular bacteria could be detected. Some isolates exhibited the classical membrane-ruffling pattern (triggering mechanism) as described for *Salmonella*, whereas other isolates showed a well-defined zipper-like mechanism found in invading Listeria. Remarkably, a high number of streptococcal isolates expressing SfbI protein induced a third, so far unknown invasion pathway, with an easily detectable morphological feature, namely the formation of large invaginations during the internalization process, sometimes looking like a “hole” in the host cell membrane (Molinari et al. 2000). Interestingly, these different invasion patterns were also detected for Group C and Group G streptococci (Haidan et al. 2000) as well as for nonencapsulated strains of *S. suis* (Benga et al. 2004) and for *S. aureus* which also invades via α5ß1 integrins (Agerer et al. 2005).

These studies revealed that streptococci also possess invasins, like the classical intracellular pathogens *Shigella*, *Listeria,* or *Salmonella*. As for the classical intracellular bacteria, streptococcal invasins aid bacteria in their internalization process into nonphagocytic host cells like fibroblasts, keratinocytes, endothelial, and epithelial cells. Invasins are surface exposed and/or diffusible proteins that can promote (i) actin rearrangement of the host cytoskeleton that stimulates engulfment of the bacteria by membrane ruffles (Dombek et al. 1999), or (ii) interact with

specific host cell receptors triggering signaling events which result in uptake of streptococci (Rezcallah et al. 2005; Ozeri et al. 1998), or (iii) co-opt host cell endocytotic pathways, caveolae, for their own internalization (Rohde et al. 2003).

The two streptococcal invasins are the streptococcal fibronectin-binding protein SfbI protein or F1 and the M proteins which are widely distributed in clinical isolates. Despite being only adhesins, these two proteins act also as invasins. SfbI/F1 protein, for example, is detectable in nearly 70–85 % of all clinical isolates and the M protein provides the basis of the Lancefield's method of *emm* gene serotyping. As mentioned before, both proteins bind to fibronectin which acts as a bridging molecule for mediating adhesion to the host cell receptor. Another member of the invasin group of proteins is the surface exposed streptococcal dehydrogenase (SDH) which is involved in the invasion of M6 serotype streptococci. SDH triggers signaling events resulting in phosphorylation of several eukaryotic proteins (Pancholi and Fischetti 1997).

3.1 SfbI/F1 Invasion of Epithelial Cells

The work of Talay et al. (2000) explained the molecular mechanism of the binding properties of the distinct domains of SfbI protein with regard to fibronectin and dissected the adherence from the invasion process. The cooperative interaction, further explained by the tandem ß-zipper mechanism (Schwarz-Linek et al. 2003), subsequently resulted in exposure of the RGD region of the fibronectin molecule allowing to bind to $\alpha_5ß_1$ integrins on the host cell surface. The interaction between the RGD region of fibronectin and integrins could be blocked by antibodies against the ß-subunit or with RGD peptides (Jadoun et al. 1998; Ozeri et al. 1998; Molinari et al. 2000). Ozeri et al. also demonstrated that the amount of bound fibronectin on the bacterial surface is responsible for the uptake efficiency into the host cell. Most properly, a certain threshold of bound fibronectin to integrins is needed to start the integrin signaling. According to the tandem ß-zipper mechanism, a single SfbI molecule is able to bind up to five fibronectin molecules. This means that the surface of SfbI-expressing streptococci is most properly covered by a high density of fibronectin. This allows streptococci to bind many integrins on the cell. A clustering of integrins underneath attached streptococci could be demonstrated by staining the ß-subunits of integrins or by a microscopic approach applying high-resolution field emission scanning electron microscopy (FESEM) to visualize integrin clustering directly on the host cell surface. Recombinant SfbI protein was coated onto 15 nm colloidal gold particles and the fate of the SfbI-gold particles on endothelial cells was observed. After 1 h, aggregates of SfbI-gold particles could be detected, meaning integrin clustering, and after 2 h SfbI-gold particle aggregates were taken up by invaginations into the cell comparable to the internalization of the SfbI-expressing *S. pyogenes isolate* (Rohde et al. 2003). This observation is in accordance with findings for other pathogens which also enter cells via integrins like *Neisseria gonorrhoeae* (van Putten et al. 1998), *S. aureus*

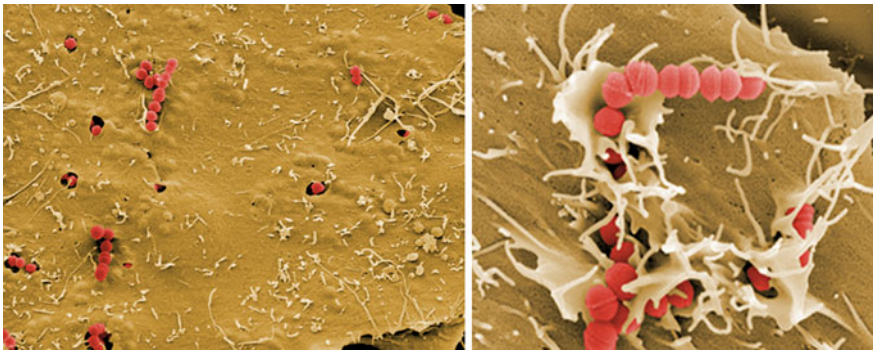

Fig. 2 FESEM image of invading Group A streptococci via SfbI-mediated fibronectin binding, integrin clustering, and formation of invaginations in the host epithelial cell (*left* image). In a mutant strain lacking SfbI protein, streptococci invade via induction of cytoskeletal rearrangements, membrane ruffles (*right* image) giving evidence that a single strain has the potential to invade via two morphological distinct pathways depending on the protein repertoire expressed on the surface

(Fowler et al. 2000), and *Yersiniae* species (Isberg and Barnes 2001). Engagement and clustering of integrin receptors is the prerequisite for sufficient uptake and subsequent integrin signaling (Isberg and Leong 1990; Isberg 1991) (Fig. 2).

In further studies, it was shown that formation of large invaginations is triggered by the SfbI-fibronectin-integrin signaling complex which is responsible for the aggregation of caveolae around adherent bacteria. Caveolae fuse with each other to form the invagination. Once inside the host cell, SfbI-expressing streptococci traffic in a new compartment called "caveosome". The intriguing aspect of this type of streptococcal invasion is the fact that caveosomes do not fuse with lysosomes. By co-opting this caveolae-mediated cellular pathway, SfbI-carrying streptococci bypass the degradation machinery of the host cell, since no fusion with lysosomes is detectable (Rohde et al. 2003).

GGS exhibit GfbA, another adhesin and invasin, on their surface which binds equal amounts of fibronectin as SfbI and interacts with $\alpha_5ß_1$ integrins. But GfbA-mediated invasion leads to the formation of membrane ruffles, i.e. rearrangement of the host cell cytoskeleton. Immunofluorescence studies demonstrated that GfbA-expressing streptococci follow the classical endocytic pathway with subsequent fusion with lysosomes to form phagolysosomes. By heterologous surface expression of GfbA in the nonpathogenic *S. gordonii,* it was demonstrated that GfbA alone is responsible for the morphologically distinct invasion mechanism compared to the SfbI-mediated caveolae dependent invasion mechanism. Sequencing of the GfbA gene demonstrated that the FnBR repeat region in the C-terminal part is very similar to SfbI. Only the N-terminal part, including the aromatic domain of GfbA (ARO) and SfbI, shows significant differences. Therefore, it was assumed that the ARO region might be responsible for the observed morphological differences in the invasion mechanism. To test this hypothesis, a

GfbA protein was constructed without the aromatic domain, and the aromatic domain in SfbI was replaced by the aromatic domain of GfbA. FESEM studies revealed that GfbA with a deleted ARO region invades like SfbI-expressing strains, whereas the SfbI strain containing the ARO region of GfbA now induced membrane ruffles. By repeating the FESEM studies for integrin clustering, it was shown that only GfbA without the aromatic domain induced integrin clustering and signaling, whereas SfbI with the ARO region of GfbA was impaired in integrin clustering and signaling. Thus, for the first time a biological function for the ARO region in a fibronectin-binding protein was demonstrated (Rohde et al. 2011).

3.2 M Protein Invasion of Epithelial Cells

For M protein-mediated invasion, fibronectin, laminin, or plaminogen/plasmin (Colognato and Yurchenco 2000; Siemens et al. 2011) binding on the bacterial surface are the prerequisites. Serotypes M1, M3, M5, M6, M12, M18, and M49 (Berkower et al. 1999; Dombeck et al. 1999; Molinari et al. 2000; Nerlich et al. 2009) have been studied with respect to HEp-2 cell invasion and all serotypes were found to be invasive. Only the highly capsulated M18 serotype was found to be less invasive because of interference of the capsule with the initial adherence process to the cells (Hagman et al. 1999).

After binding to fibronectin and $\alpha_5ß_1$-integrins, the serotype M1 promotes invasion of human lung epithelial cells. A M1 negative mutant had a significant reduced invasion capability. M1 protein can also bind to laminin with subsequent internalization in host cells (Cue et al. 1998). The mechanism of entrance was accompanied by cytoskeletal rearrangements and represented a zipper-like mechanism in HeLa cells as shown by scanning EM (Dombek et al. 1999). This observation is in contrast to the caveolae-mediated internalization of SfbI/F1 carrying strains, despite the fact that also M1 serotype strains bind fibronectin and subsequently $\alpha_5ß_1$ integrins.

The zipper-like invasion was mediated by an early intimate contact of streptococci with host cell microvilli and invading streptococci were associated with polymerized actin at their site of entry. Identical observations of actin polymerization underneath the entry port were observed for a M5 serotype/A8 strain (Molinari et al. 2000) which does not express SfbI. M1 protein-mediated invasion subsequently leads to the fusion with lysosomes to form a phagolysosome.

4 Dissemination and Persistence

After a first episode of infection with streptococci, a small percentage of patients will experience recurrent tonsillitis or erysipelas episodes. These recurrent infections may be attributed to a bacterial subpopulation surviving beyond the first

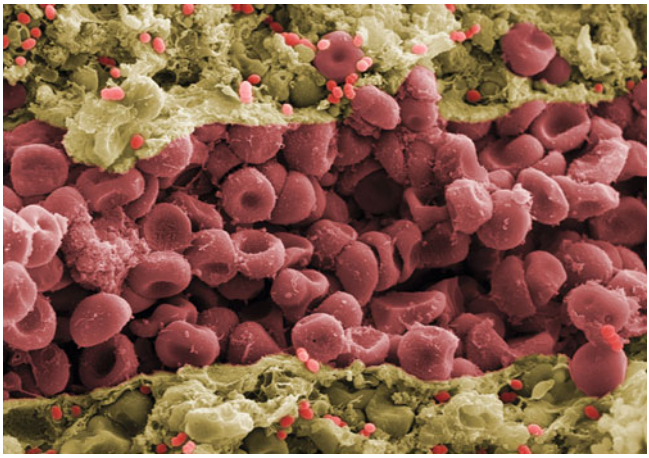

Fig. 3 FESEM image of dissemination of Group A streptococci. Streptococci were administered intravenously. From the blood stream, streptococci passed through the endothelial layer of the blood vessels into deeper tissue (streptococci in *pink*, *red blood* cells in *red*)

episode. There are two plausible reasons why the streptococci might persist: (i) by hiding in the matrix proteins of the human mucosa, especially serotype M3 and M18 by binding to collagen, (ii) or intracellularly by residing in an intracellular compartment or in the cytoplasm (Fig. 3).

For dissemination in the host, two possible ways are conceivable. Firstly, streptococci might invade certain cells and travel unrecognized in these cells through the body until reaching a safe niche in the body—Trojan horse theory. Secondly, after bacteraemia streptococci might be able to passage through the dense endothelial cell layer of the blood vessels to gain access into deeper tissue. For both ways, first experimental evidence has been obtained during recent years. Nevertheless, we are far away from understanding the entire multiple strategies of streptococci during dissemination in the body. For example, it was shown that GAS, which are resistant to killing in PMNs, may exploit the ability of the neutrophils to transmigrate through endothelial cell barrier of the vascular system for spread via the blood stream (Medina et al. 2003a, b). Surprisingly, streptococci were also found residing in macrophages when biopsies of patients with soft tissue infections were examined, supporting the Trojan horse theory (Thulin et al. 2006). These studies conclusively showed that host phagocytic cells, especially macrophages, were the reservoir for intracellular GAS during infection. Remarkably, the authors found evidence that localization of GAS varied and depended on the severity of tissue infection. Intracellular streptococci were predominantly found in noninflamed tissue with a low bacterial load, whereas in inflamed tissue, even after prolonged intravenous treatment with antibiotics, high loads of GAS were detected. It has been suggested that internalization could indeed promote the spread of GAS within the tissue. Macrophages might then play the role of the Trojan horse

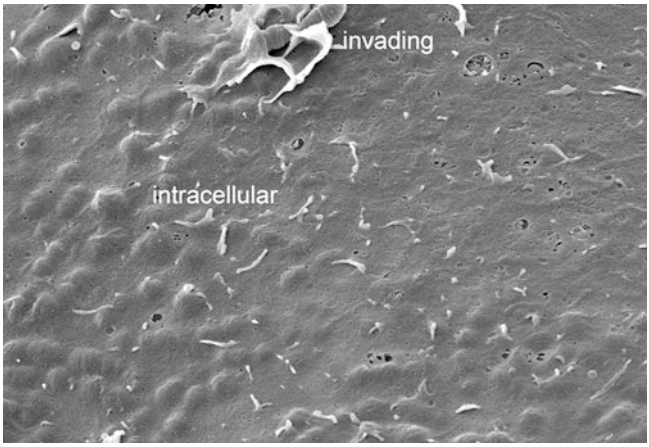

Fig. 4 FESEM image of a highly invasive Group G isolate, *Streptococcus dysgalactiae* subspec. *equisimilis,* from an aneurysm of a patient. Despite antibiotic treatment, this strain was persistent for several years

in the infected tissue. On the other hand, work by Cywes et al. (2000) described another type of streptococcal invasion. They demonstrated that binding to CD44 on a confluent layer of human keratinocytes via HA (capsule) of encapsulated strains induced cytoskeletal changes, membrane ruffling, and a disruption of cellular junctions after binding to the host cell lamellipodia. In contrast, nonencapsulated strains attached to host membrane regions devoid of lamellipodia. This type of invasion opens up the way for streptococci to gain access into deeper tissue layers. For Group B streptococci (GBS), an identical paracellular translocation through the epithelial barrier during onset of infection has been reported. Soriani et al. (2008) applied differentiated epithelial cells grown in cell culture transwell inserts for their studies. Translocation of bacteria occurred without measurable decrease in the transepithelial resistance and analysis of ultra-thin sections revealed an intimate association of GBS with the intercellular junctions (Soriani et al. 2008). Recently, a third mechanism has been found demonstrating that streptococci mediate an exocytosis process in which streptococci residing in a phagolysosome can trigger their own exocytosis process, with Rab27 being involved, to transmigrate through an endothelial barrier (Talay et al. personal communication).

For persistence in the host, only scant data are available. For example, *S. dysgalactiae* subsp. *equisimilis* (Group C or Group D) are relatively common in invasive infections, especially in older patients (Sylvetsky et al. 2002). This species (type stG485.0) was involved in several recurrent bacteremic sepsis episodes despite prolonged antibiotic treatment over years and using opsonizing antibodies against the isolate. The isolate was suspected to reside in an aortic aneurysm of the patient. With a cell culturing model, it was shown that the isolate possesses a very high invasiveness in endothelial HUVEC cells (Rohde et al. 2012) (Fig. 4).

That streptococci actually can survive for a long period of time was shown in batch cultures using sugar-limited Todd-Hewitt broth. Bacteria were still viable and culturable after 1 year (Wood et al. 2005). Other experiments extended the survival time to over 45 months. An overnight culture with an inoculation with 5 μl of the sedimented bacteria, grown in TBS, was sufficient to obtain fully viable streptococci. Even more surprising was the fact that FESEM imaging of the bacteria revealed no visible difference of 45-month-old streptococci to the overnight regrown bacteria; only the interior morphology of the bacteria was remarkably different (Rohde, unpublished). It is speculated that during persistence *S. pyogenes* might enter a kind of a quiescent state, due to conditions which do not support rapid bacterial growth, such as nutrition limitations. If a trauma from outside destroys areas of intracellular streptococci containing tissue bacteria, allowing access to new nutrition due to infiltration of immune cells etc., to the damaged tissue side, then bacteria can respond with a fulminant growth thereby causing a recurrent infection.

5 Conclusions

Adhesion of streptococci through the multiple adhesins and internalization into human epithelial and endothelial cells by invasins creates a safe sanctuary for streptococci protecting them against the host immune system and antibiotic treatment. Together, with the capability to penetrate cellular barrier for dissemination and hijacking immune cells like PMNs for their own spread in the body makes streptococci a very versatile and not easy to eradicate human pathogen. Since the work of Österlund and colleagues (Oesterlund and Engstrand 1997; Oesterlund et al. 1997), who provided first evidence for the existence of persisting intracellular streptococci, evidence has accumulated that streptococcal invasion into host cells is a severe therapeutic problem (Thulin et al. 2006; Rohde et al. 2012). The routinely used β-lactam antibiotics, penicillins, are not efficiently permeating into eukaryotic cells and fail to completely eradicate intracellular GAS. Recently, macrolides like erythromycin or derivatives thereof or an azalide, azithromycin were found to be more efficient in eradicating intercellular streptococci in vitro and may present an attractive therapeutic alternative for eradication of streptococci (Kaplan et al. 2006). Nevertheless, the use of macrolides faces another drawback since streptococci have developed a strong resistance against macrolides in some countries. The mostly in vitro obtained observations of streptococcal adhesion, internalization, trafficking inside the cell, dissemination, and persistence, taken together with in vivo studies on persistence of streptococci, should allow for a more effective approach for clinical management of Group A *S. pyogenes* carriers with the aim to eradicate bacteria at the onset of the infection.

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