Binding of the *Streptococcus gordonii* DL1 Surface Protein Hsa to the Host Cell Membrane Glycoproteins CD11b, CD43, and CD50

Yumiko Urano-Tashiro,* Ayako Yajima, Eizo Takashima, Yukihiro Takahashi, and Kiyoshi Konishi

Department of Microbiology, School of Life Dentistry at Tokyo, Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan

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Infective endocarditis is frequently attributed to oral streptococci. The mechanisms of pathogenesis, however, are not well understood, although interaction between streptococci and phagocytes are thought to be very important. A highly expressed surface component of *Streptococcus gordonii* DL1, Hsa, which has sialic acid-binding activity, contributes to infective endocarditis in vivo. In the present study, we found that *S. gordonii* DL1 binds to HL-60 cells differentiated into monocytes, granulocytes, and macrophages. Using a glutathione S-transferase (GST) fusion to the NR2 domain, which is the sialic acid-binding region of Hsa, we confirmed that the Hsa NR2 domain also binds to differentiated HL-60 cells. To identify which sialoglycoproteins on the surface of differentiated HL-60 cells are receptors for Hsa, intrinsic membrane proteins were assessed by bacterial overlay and far-Western blotting. *S. gordonii* DL1 adhered to 100- to 150-kDa proteins, a reaction that was abolished by neuraminidase treatment. These sialoglycoproteins were identified as CD11b, CD43, and CD50 by GST pull-down assay and immunoprecipitation with each specific monoclonal antibody. These data suggest that *S. gordonii* DL1 Hsa specifically binds to three glycoproteins as receptors and that this interaction may be the initial bacterial binding step in infective endocarditis by oral streptococci.

*Streptococcus gordonii* and related species of viridans group streptococci are components of the normal microbial flora of the human oral cavity (19, 20, 23). These organisms play significant roles as pioneer colonizers in the development of dental plaque (35). In addition, these streptococci are also well known for their ability to colonize damaged heart valves and are the most frequently identified bacteria as primary etiological agents of infective endocarditis (2, 3, 14).

*S. gordonii*, *Streptococcus sanguinis*, and *Streptococcus oralis* adhere to saliva-coated hydroxyapatite, an experimental model of the tooth surface, and attach to host cells such as erythrocytes, platelets, and polymorphonuclear leukocytes (PMNs) (18, 19, 21, 39, 46). A common mechanism involved in these interactions is the recognition of surface-associated host sialo-glycoconjugates. Recently, such interactions have been found to involve the binding of streptococcal adhesins identified as large serine-rich glycoproteins (6, 33, 47) to membrane sialoglycoproteins of host cells (6, 33, 49, 52). We previously reported that the *S. gordonii* DL1 *hsa* gene encodes a large serine-rich repeat protein (Hsa) composed of 2,178 amino acid residues. Hsa consists of an N-terminal nonrepetitive region (NR1), a serine-rich repeat region (SR1), another nonrepetitive region (NR2), an additional serine-rich repeat region (SR2), and a C-terminal cell wall anchoring domain (47). NR2 of Hsa is considered to be a binding site for α2-3-linked sialic acid (46, 47, 49). SR2, which accounts for over 75% of the length of Hsa, is a glycosylated region containing GlcNAc (46, 49). This glycosylation may confer an extended rod-shaped conformation on the serine-rich region, enabling this region to function as a molecular stalk for cell surface presentation of the putative amino-terminal receptor-binding domain (49).

Hsa binds to the α2-3-linked sialic acid termini of O-glycosylated mucin-type glycoproteins, including salivary mucin MG2, platelet glycoprotein Ib (GPIbα), and leukosialin, the major surface glycoprotein of human PMNs (7, 39, 40, 47, 48, 49, 52). Moreover, fibronectin and GPIIb, another platelet sialoglycoprotein, have been identified as receptors for Hsa (24, 52). Hsa of *S. gordonii* DL1 and SraP, a Hsa homologue of *Staphylococcus aureus*, contribute to infective endocarditis in vivo (41, 50). The binding of streptococci to human platelets is thought to be a major virulence determinant in the pathogenesis of infective endocarditis (22). On the other hand, significant differences in the virulence of representative *S. gordonii* strains in the rat model of infective endocarditis do not appear to be correlated with the adhesion of these bacteria to isolated platelets or the fibrin-platelet matrix but instead are correlated with the biological consequence of bacterial binding to PMNs (54). The latter finding suggests that the ability of *S. gordonii* to survive in PMNs following adhesin-mediated phagocytosis may be an important virulence determinant of infective endocarditis. The mechanism by which streptococci escape from the immune response, including phagocytosis, during the progression of infective endocarditis is not well understood.

In the present study, we showed that *S. gordonii* DL1 interacts with phagocytes such as monocytes, granulocytes, and macrophages. Furthermore, we identified the receptors bound to *S. gordonii* DL1 Hsa. Our data strongly suggest that CD11b, CD43, and CD50 are the host receptors for *S. gordonii* DL1 Hsa.

**MATERIALS AND METHODS**

**Cell culture.** The human promyelocytic leukemia HL-60 cell line was maintained in RPMI 1640 medium with 10% fetal calf serum. For the differentiation assay, 2 × 10⁵ HL-60 cells/ml were treated with either 100 nM 1α,25-dihydroxyvitamin D₃ (VD₃) (Calbiochem, Darmstadt, Germany) for 24 h, 1 μM all-trans...
retinoic acid (RA; Sigma-Aldrich, St. Louis, MO) for 4 days after the addition of 1.25% dimethyl sulfoxide (DMSO) for 16 h, or 32 nM 12-β-tetradecanoylphor- bol-13-acetate (TPA; Sigma-Aldrich) for 2 days. Cells were cultured under 5% CO2 at 37°C. Myeloperoxidase and nonspecific esterase staining were performed as markers of differentiation to granulocytes and to monocytes/macrophages, respectively, as described previously (10, 27; data not shown). In addition, plastic adherence was confirmed to distinguish macrophages from monocytes (10, 38; data not shown).

**Bacteria.** The *S. gordonii* strains used in the present study were DL1 (wild type) and its mutant EM230 (DL1 hsa:erm4M) (46, 47). Streptococci were cultured overnight at 37°C in brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ).

**Attachment of bacteria to HL-60 cells.** Glutathione S-transferase (GST) and GST-HsNr2 were prepared as described previously (53). All bacteria were washed three times with 1× phosphate-buffered saline (PBS). HL-60 cell preparations (10⁶ cells/ml) were challenged with bacteria (10⁵ cells/ml) for 2 h at 37°C. Cells were washed twice with 1× PBS containing 1% bovine serum albumin (BSA) and stained by using the Wright-Giemsa method (protocol HEMAM3; Biochemical Sciences, Swedenboro, NJ). Bacterial binding to HL-60 cells was quantitatively evaluated by counting the bacterial cell number on the surface of 30 HL-60 cells, which were randomly selected. Statistical differences in the mean of the bound bacterial cell numbers were evaluated by the unpaired Welch’s *t* test. For the binding inhibition assay, cells were pretreated with various concentrations of GST or GST-HsNr2 for 30 min at 37°C.

**Cell extracts and SDS-PAGE.** Cells were lysed containing 20 mM Tris-HCl (pH 7.8)–150 mM NaCl (TBS) containing 5 mM MgCl₂ and 0.1% Nonidet P-40. Aliquots of 20 μg of cell lysate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4 to 12% gradient gel (Invitrogen, Carlsbad, CA). The proteins were electroeluted transferred to a nitrocellulose membrane (Millipore, Billerica, MA).

**Bacterial overlay.** *S. gordonii* DL1 at 2 × 10⁶ cells/ml in 1× PBS were bioin labeled by incubation with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC)-biotin (Pierce, Rockford, IL) at 100 μg/ml for 1 h at room temperature. Untreated nitrocellulose transfers, or transfers incubated with 1 μg of neuraminidase (Sigma-Aldrich) in 1× PBS for 1 h at 37°C, were blocked in TBS containing 5% BSA, 1 mM CaCl₂, 1 mM MgCl₂, and 0.02% sodium azide for 4 h at room temperature. Labeled bacteria were added to a final concentration of 8 × 10⁷ cells/ml. The overlays were incubated overnight at 4°C without mixing and washed four times at room temperature for 5 min with TBS containing 0.05% Tween 20, 1 mM CaCl₂, 1 mM MgCl₂, and 0.02% sodium azide. The blots were then incubated with 0.2 U of avidin-D-alkaline phosphatase (Vector, Burlingame, CA) per ml in the same buffer for 30 min, washed three times for 5 min, and developed with BCIP (5-bromo-4-chloro-3-indolylphosphate) and nitroblue tetrazolium. That no reactive bands were observed without the labeled bacteria was confirmed prior to the experiment.

**Far-Western blotting.** Neuraminidase-treated or untreated nitrocellulose transfers were blocked in 1× blocking reagent (Roche Diagnostics, Indianapolis, IN) in 1× PBS for 4 h at room temperature and then incubated overnight at 4°C with 0.1 μM GST or GST-HsNr2. After three washes with 1× blocking reagent, the transfers were incubated for 1 h with anti-GST antibody (1:1,000; GE Healthcare Bioscience, Uppsala, Sweden). Antibody binding was detected by incubating the transfers for 1 h with horseradish peroxidase (HSP)-conjugated anti-goat immunoglobulin G (IgG) (1:2,000; Bio-Rad, Hercules, CA), followed by development using the SuperSignal chemiluminescent detection system (Pierce).

**Western blotting.** The membranes were blocked in TBS containing 0.01% Tween 20 and 1% BSA for 1 h at room temperature and incubated overnight at 4°C with anti-GST, anti-Cd43 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cd50 (1:250; R&D Systems, Minneapolis, MN), or anti-actin (1:1,000; Sigma-Aldrich). After three washes with TBS, the membranes were incubated with either HRP-conjugated anti-mouse IgG (1:2,000; Vector), HRP-conjugated anti-goat IgG, or HRP-conjugated anti-rabbit IgG (1:2,000; Vector) as indicated. Signals were visualized with the SuperSignal chemiluminescent detection system (Pierce).

**GST pull-down assay.** The cells were lysed with TMN buffer. The GST pull-down assay was performed according to the method of Yamaji et al. (53). Western blotting analysis was performed as described above with the appropriate antibodies.

**Immunoprecipitation.** The cells were lysed with TMN buffer. For immunoprecipitations, equal amounts of lysate proteins and protein A-Sepharose beads (GE Healthcare) charged with anti-Cd11b antibody were incubated at 4°C overnight. The beads were washed twice with TBS. Immunoprecipitated proteins or cell lysates were mixed with 2× Tris-glycine SDS sample buffer (Invitrogen) and were separated by SDS-PAGE. Western blotting analysis was performed as described above with the appropriate antibodies.

**RESULTS**

**Attachment of *S. gordonii* DL1 to HL-60 cells.** HL-60 cells can be induced to terminally differentiate into granulocytes, monocytes, or macrophages in response to various inducers (10). As indicated in Fig. 1, VD₃, DMSO plus RA, and TPA induced HL-60 cells to differentiate into monocytes (Fig. 1Ac and d), granulocytes (Fig. 1Ae and f), and macrophages (Fig. 1Ag and h), respectively (4, 12, 55). To determine the ability of *S. gordonii* DL1 to adhere to human peripheral leukocytes, HL-60 cells were differentiated into monocytes, granulocytes, or macrophages, and the cells were treated for 2 h with *S. gordonii* DL1 at a ratio of 100 bacterial cells per host cell. *S. gordonii* DL1 bound to undifferentiated and differentiated...
HL-60 cells (Fig. 1Aa, c, e, and g). In contrast, when HL-60 cells were incubated with EM230, an hsa insertion mutant, no bacterial cell binding to HL-60 cells was observed (Fig. 1Ab, d, f, and h). All of these differences were statistically significant ($P < 10^{-9}$) (Fig. 1B). These results indicate that S. gordonii DL1 binds to any of these types of leukocytes and that this binding ability is mediated by Hsa.

To confirm whether Hsa is involved in bacterium-host cell interactions, HL-60 cells were incubated with GST or GST-HsaNR2 protein, followed by the bacterial cell binding assay. As shown in Fig. 2, binding of S. gordonii DL1 to HL-60 cells was significantly inhibited by GST-HsaNR2 (Fig. 2Ae, f, and g), but not by GST (Fig. 2Aa, b, and c) ($P < 10^{-9}$, Fig. 2B). These results confirm that Hsa plays an essential role in bacterium-host cell interactions.

**Detection of HL-60 cell receptors for S. gordonii DL1 Hsa.** To identify HL-60 cell receptors for S. gordonii DL1, transfers of HL-60 cell extracts were overlaid with biotinylated bacteria (Fig. 3). Fetuin, which has NeuAc$_2$-3Gal$_1$-3GalNac termini (44), was used as a positive control. Bands of approximately 100 to 150 kDa were specifically detected by S. gordonii DL1 but were poorly detected by EM230 (Fig. 3, upper panels). We confirmed that bacterial binding to HL-60 cell receptors occurs in a sialic-acid-dependent manner, since Hsa has been shown to bind to sialoglycoprotein (47). Transfers of HL-60 cell extracts were treated with neuraminidase and then overlaid with biotinylated bacteria. As shown in Fig. 3 (lower panel), neuraminidase treatment of the transfer markedly reduced the binding of S. gordonii DL1. To further verify whether Hsa is involved in bacterial binding to the 100- to 150-kDa proteins, transfers of HL-60 cell extracts were analyzed by far-Western blotting with GST or GST-HsaNR2 (Fig. 4). When probed with GST-HsaNR2, the fusion protein, like S. gordonii DL1, bound to the 100- to 150-kDa proteins (Fig. 4, upper left panel). In control experiments, GST alone did not bind to any proteins (Fig. 4, upper right panel). The fusion protein binding was abolished by pretreatment of an identical blot with neuraminidase (Fig. 4, lower panel). These results suggest that S. gordonii DL1 binds to the 100- to 150-kDa proteins of HL-60 cells via interaction with Hsa and that the binding of Hsa with HL-60 cell receptors is indeed dependent on sialic acid.

**Hsa binds to CD11b, CD43, and CD50.** CD43 (also known as leukosialin or sialophorin), which has a molecular mass of approximately 95 to 135 kDa, is an O-linked sialoglycoprotein (17). Another glycoprotein, CD50 (also known as intercellular adhesion molecule-3), which has a molecular mass of 110 to 140 kDa, contains 15 potential N-linked glycosylation sites (42). In both the undifferentiated and the differentiated HL-60 cells, CD43 was detected as 100- to 150-kDa bands (Fig. 5A, upper panel), and CD50 was identified as 110- to 140-kDa bands (Fig. 5A, middle panel). Thus, we considered that the

**FIG. 3.** Bacterial overlays on nitrocellulose transfers of extracts of HL-60 cells. Cellular extracts from undifferentiated or differentiated HL-60 cells were separated by SDS-PAGE and transferred to nitrocellulose. Untreated or neuraminidase-treated transfers were overlaid with either biotinylated S. gordonii DL1 or EM230 and washed. Bound biotinylated bacteria were detected with avidin-D-alkaline phosphatase. Fetuin or asialofetuin (5 μg per well) were included as controls. The positions of the molecular mass markers are indicated on the left in kilodaltons. The positions of approximately 100- to 150-kDa bands specifically detected by S. gordonii DL1 but not by EM230 are indicated on the right by a vertical bar.

**FIG. 2.** Inhibition of bacterial binding to HL-60 cells by GST-HsaNR2 protein. (A) HL-60 cells were either untreated (–, d) or treated with various concentrations of GST (a, b, and c) or GST-HsaNR2 (e, f, and g) protein for 30 min at 37°C. Cells were then incubated with S. gordonii DL1 for 2 h at 37°C. Bacterial binding was determined microscopically using Wright-Giemsa staining. (B) The means and standard deviations ($n = 30$) of bacterial cell numbers bound to a HL-60 cell are indicated. *, $P < 10^{-9}$. 
100- to 150-kDa bands may be CD43 and CD50 on the basis of their characteristic glycosylation pattern, reported molecular mass, and reactivity with specific antibodies as indicated in Fig. 5A. To verify the interaction between Hsa and these glycoproteins, GST pull-down assays were performed. GST-HsaNR2 or GST was incubated with extracts of undifferentiated and differentiated HL-60 cells and pull-down complexes were obtained by using glutathione-Sepharose 4B beads, followed by Western blotting with anti-CD43 antibody and anti-CD50 antibody (Fig. 5B). Proteins of approximately 100 to 150 kDa were specifically co-pulled-down with GST-HsaNR2 (Fig. 5B, upper panel). By using anti-CD43 antibody, we found that CD43 bound to GST-HsaNR2 but not GST in undifferentiated and differentiated HL-60 cells. Interestingly, CD50 bound to GST-HsaNR2 only in extracts of DMSO plus RA-treated HL-60 cells (Fig. 5B, middle panel).

In addition to CD43 and CD50, we found another HL-60 cell membrane protein is a receptor for Hsa. CD11b, which has a molecular mass of 165 kDa, is a leukocyte adhesion heterodimeric glycoprotein (CD11b/CD18) with 19 N-linked glycosylation sites (12). To clarify the interaction between Hsa and CD11b, extracts of undifferentiated and differentiated HL-60 cells were immunoprecipitated with anti-CD11b antibody, followed by far-Western blotting with GST-HsaNR2 protein (Fig. 6). A 165-kDa band was clearly detected following concentration by immunoprecipitation with anti-CD11b antibody (Fig. 6, upper panel). As shown in Fig. 6 (lower panels), the binding of GST-HsaNR2 to CD11b was observed in immunoprecipitates with anti-CD11b antibody, although the positive bands in HL-60 and VD3 were rather faint. Taken together, these results indicate that CD11b, CD43, and CD50 are the receptors for S. gordonii DL1 Hsa.

**DISCUSSION**

The results of this study demonstrate that S. gordonii DL1 is attached to monocytes, granulocytes, and macrophages and that the binding ability of S. gordonii DL1 to these cells is mediated by Hsa. Furthermore, the results clearly indicate that the cell surface glycoproteins CD11b, CD43, and CD50 are the receptors for Hsa and that the NR2 region of Hsa is bound to sialic acid moieties of receptors on the cell.

Hsa may have an extended rod-shaped conformation, facilitating the presentation of the Hsa receptor-binding domain at the periphery of the bacterial surface (46, 47, 49). Therefore, the recognition of the sialic acid of these host cells by Hsa may play the most important role in the primary contact between S. gordonii bacterial cells and the host cells.

CD43 is a major sialoglycoprotein expressed widely in various leukocytes such as granulocytes, monocytes, macrophages, and T lymphocytes. CD43 contains 70 to 80 O-linked oligosac-
macrophages, including phagocytosis, transmigrational adhesion, nitric oxide production, generation of a respiratory burst, and the production of interleukin-12 (1, 11, 26, 29, 31, 36, 37, 45). In addition, CD11b binds to lipopolysaccharide and then activated transcription factor NF-κB (16). The identification of CD43 and CD11b as host cell receptors for S. gordonii DL1 Hsa provides a strong basis for further exploration of the signaling processes triggered by these bacteria and their effects on host cell function.

Bacteremias are usually cleared by the reticuloendothelial system within minutes. Enteric gram-negative bacteria frequently cause bacteremias but rarely cause endocarditis. In contrast, oral streptococci are infrequently isolated from dental trauma-induced bacteremia (34), but they are frequently identified from sites of native valve endocarditis in humans (5, 51). It appears that oral streptococci can uniquely avoid clearance by the reticuloendothelial system and are capable of adhering to and proliferating in endocardial vegetation. Young Lee et al. reported that the S. gordonii strains which cause severe endocarditis were either resistant to PMN-dependent killing or the number of these cells was reduced only by ca. 50% (54). In addition, the virulence of S. gordonii DL1 in catheterized rats was significantly reduced by deletion of hsa (50). Thus, S. gordonii DL1 Hsa may be involved in the resistance of bacteria to PMN-dependent killing.

Unidentified molecules that do not contain sialic acid appear to bind to S. gordonii (Fig. 3), suggesting that such unsialylated host receptors and multiple adhesins, such as ScA (28), homologues of Streptococcus parasangusius FimA (8), SspA/SspB (25), streptococcal antigen I/II family, and CshA (30) of S. gordonii may also mediate the bacterial binding to the host cells. In future studies, we will examine the association of Hsa and other adhesins with resistance to PMN-dependent killing of S. gordonii. The results of such studies may provide important insights into the pathogenesis of infective endocarditis induced by the oral viridans group streptococci.

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