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Research Paper

A Critical Role of Zinc Importer AdcABC in Group A *Streptococcus*-Host Interactions During Infection and Its Implications for Vaccine Development

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ABSTRACT

Bacterial pathogens must overcome host immune mechanisms to acquire micronutrients for successful replication and infection. *Streptococcus pyogenes*, also known as group A streptococcus (GAS), is a human pathogen that causes a variety of clinical manifestations, and disease prevention is hampered by lack of a human GAS vaccine. Herein, we report that the mammalian host recruits calprotectin (CP) to GAS infection sites and retards bacterial growth by zinc limitation. However, a GAS-encoded zinc importer and a nuanced zinc sensor aid bacterial defense against CP-mediated growth inhibition and contribute to GAS virulence. Immunization of mice with the extracellular component of the zinc importer confers protection against systemic GAS challenge. Together, we identified a key early stage host-GAS interaction and translated that knowledge into a novel vaccine strategy against GAS infection. Furthermore, we provided evidence that a similar struggle for zinc may occur during other streptococcal infections, which raises the possibility of a broad-spectrum prophylactic strategy against multiple streptococcal pathogens.

1. Introduction

Nutrient acquisition by pathogens during infection is imperative for bacterial proliferation and disease development (Porcheron et al., 2016). The host exploits the nutritional requirements of the pathogen to limit growth by altering the availability of micronutrients at the colonization surface in a process referred to as nutritional immunity (Corbin et al., 2008; Damo et al., 2013; Kehl-Fie and Skaar, 2010; Ong et al., 2014). Although insights into the role of nutritional immunity in host defenses against pathogenic microorganisms are rapidly emerging (Corbin et al., 2008; Damo et al., 2013; Diaz-Ochoa et al., 2016; Gaddy et al., 2014; Haley et al., 2015; Hood et al., 2012; Liu et al., 2012), its involvement against several human pathogens, including group A *streptococcus* (GAS), is poorly understood. GAS is a versatile human-specific pathogen that colonizes different anatomic sites and causes diverse disease manifestations (Carapetis et al., 2005; Cunningham, 2000; Olsen et al., 2009). Furthermore, recurring and untreated infections can lead to post-infection immune-mediated complications such as rheumatic heart disease and post-streptococcal glomerulonephritis (Carapetis et al., 2016; Maurice, 2013; Rodriguez-Iturbe and Batsford, 2007). Given the morbidity and mortality associated with GAS infections, and the lack of clinically available prophylactic measures, identification of novel vaccine or antimicrobial targets to treat GAS infections is imperative (Carapetis et al., 2005; Carapetis et al., 2016; Sheel et al., 2016; Steer et al., 2016, 2013).

Zinc is an essential nutrient for microbial growth, but can be toxic in excess (Blencowe and Morby, 2003; Coleman, 1998). The delicate balance between zinc sufficiency and toxicity is maintained by bacterial metal homeostasis systems (Klaus, 2005; Moore and Helmann, 2005). The GAS metalloregulator, adhesin competence repressor (AdcR), regulates zinc homeostasis by monitoring the intracellular zinc concentration and modulating GAS adaptive response to zinc limitation (Sanson et al., 2015). During zinc sufficiency, the zinc-bound AdcR represses the expression of target genes, whereas the apo-AdcR relieves the repression during zinc deficiency. When cells encounter zinc limitation, GAS upregulates genes encoding zinc acquisition systems (*adcA, adcAII/lmb, adcBC, phtD*, and *phtY*), and zinc sparing responses (*rpsN.2* and *adh*) (Sanson et al., 2015). The primary GAS zinc uptake system, AdcABC, is composed of a cell surface-exposed zinc-binding protein (AdcA), an inner membrane permease (AdcB), and a cytosolic ATPase (AdcC) that provides the energy for zinc import by ATP hydrolysis. GAS also employs additional factors such as AdcAII/Lmb, PhtD, and PhtY to overcome zinc limitation (Sanson et al., 2015). Both AdcA and AdcAII share homologous N-terminal ZnuA-like extracellular zinc-binding domains. However, AdcA has an additional C-terminal ZinT-like

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domain and the role of the ZinT-like domain of AdcA in GAS homeostasis is yet to be elucidated. Although AdcAII/Lmb and Pht proteins contribute to streptococcal adaptive responses to zinc limitation (Bayle et al., 2011; Moulin et al., 2016; Tedde et al., 2016), the exact mechanisms by which they facilitate zinc acquisition remain unknown. Nevertheless, the significance of the AdcR signaling pathway to GAS pathogenesis is underscored by the observation that inactivation of *adcR* caused dysregulation of zinc homeostasis and significantly attenuated GAS virulence (Sanson et al., 2015).

The host recruits calprotectin (CP), an S100A8/A9 heterodimer, at microbial colonization surfaces and inhibits bacterial proliferation by sequestration of zinc, and manganese (Corbin et al., 2008; Damo et al., 2013; Diaz-Ochoa et al., 2016; Liu et al., 2012; Lusitani et al., 2003). The antibacterial activity of CP against bacterial and fungal pathogens has been demonstrated (Corbin et al., 2008; Damo et al., 2013; Diaz-Ochoa et al., 2016; Liu et al., 2012; Sohnle, et al., 1991; Urban, et al., 2009), but its role against most of the streptococcal pathogens is unknown. Emerging evidence indicates that GAS encounters host-mediated zinc immune mechanisms (Brenot et al., 2007; Ong et al., 2014). However, molecular details underlying host defense mechanisms, bacterial countermeasures, and their role in GAS pathogenesis are lacking. Using a multidisciplinary approach, we discovered that CP is a major host defense factor against GAS infection in different niches and mediates growth inhibition primarily by zinc sequestration. Conversely, GAS employs a high-affinity zinc uptake system and a refined sensory system to overcome CP-mediated growth inhibition. To realize the translational potential of our findings, we assessed and validated the extracellular component of the zinc importer, AdcA, as a potential GAS vaccine candidate.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. Strain MGAS10870 is representative of serotype M3 strains that cause invasive infections whose genome has been fully sequenced and has wild-type sequences for all major regulatory genes (Beres et al., 2010). Details of isogenic mutants construction using the parental serotype MGAS10870 is described in the Supplemental Experimental Procedures. *Escherichia coli* DH5α strain was used as the host for plasmid constructions and BL21(DE3) strain was used for recombinant protein overexpression. GAS was grown routinely on Trypticase Soy agar containing 5% sheep blood (BSA; Becton Dickinson) or in Todd-Hewitt broth containing 0.2% (w/v) yeast extract (THY; DIFCO). The *Escherichia coli* was grown in Luria-Bertani broth (LB broth; Fisher Scientific).

2.2. Animal Infection Studies

Mouse experiments were performed according to protocols approved by the Houston Methodist Hospital Research Institute Institutional Animal Care and Use Committee. These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, 8th edition. Mouse infections studies, analysis of colony-forming units (CFU), and histopathology of the infected tissues were carried out as detailed in the Supplemental Experimental Procedures.

2.3. Preparation of Total Protein Extracts From the Infected Tissues

To ensure that the CP levels measured are predominantly the secreted fraction, not the neutrophil cytosolic fraction, we employed methods to minimize contamination with intact neutrophils, neutrophil lysis, and acquisition of cytosolic CP. Typically, the abscess fluid is mostly acellular, and contains predominantly necrotized neutrophils. Intact neutrophils are likely to be around the edges of abscesses bordering with the healthy tissues and were not included in the sample preparation. The abscess fluid from the GAS infected tissues were carefully aspirated by puncturing the lesion and the purulent fluid was resuspended (30 mg of lesion/ml) in sterile PBS supplemented with a protease inhibitor cocktail pellet. Subsequently, the lesions were homogenized gently on ice and cell debris was removed by centrifugation at 20,000 $\times g$ for 30 min. The clarified supernatant was collected, filtered with 0.22 μm filter, and the total protein concentration was assessed by Bradford assay. The supernatant containing equal total protein concentration was assayed for CP by immunoblotting and ELISA assays.

2.4. Identification of Calprotectin in Infected Tissues by Immunoblotting

Equal concentrations of the total protein extracts from the tissue samples were resolved on a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with rat monoclonal anti-mouse S100A8, S100A9 (R&D Systems), and polyclonal anti-mouse GAPDH (Invitrogen) antibodies. Detection was accomplished by goat anti-rat secondary antibody conjugated with horseradish peroxidase (R&D Systems), and visualized by chemiluminescence.

2.5. Quantification of Calprotectin in Infected Tissues by ELISA

ELISA assays to measure calprotectin levels in the murine tissues were performed according to the manufacturer's instructions (Hycult Biotech). The calibration curve was generated using the calprotectin standard in the kit, and this curve was used to derive the concentration of calprotectin in the tissue samples. Samples were analyzed in triplicates and data were graphed as calprotectin concentration in micrograms per ml of total protein extract.

2.6. Bacterial Growth Studies in the Presence of CP

Recombinant human calprotectin was overexpressed and purified as detailed in Supplemental Experimental Procedures (Fig. S1). A 1:100 dilution of overnight GAS growth was inoculated into THY broth supplemented with calprotectin buffer (20 mM Tris pH 7.5, 0.1 M NaCl, 10 mM β-mercaptoethanol, 3 mM $CaCl_2$). The indicated concentrations of CP were added to the starter culture and growth was monitored by measuring A600 with a microplate reader. Samples were analyzed in triplicate and at least two different CP preparations were used.

2.7. Metal Content Analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Metal concentration in CP-treated growth medium, intracellular metal content of different GAS strains and variants of recombinant AdcR were analyzed by ICP-MS as described in Supplemental Experimental Procedures.

2.8. Transcript Level Analysis

GAS strains were grown to the indicated OD_{600} and incubated with two volumes of RNAprotect (Qiagen) for 10 min at room temperature. RNA isolation and purification were performed using an RNeasy kit (Qiagen). cDNA was synthesized from the purified RNA using Superscript III (Invitrogen) and Taqman quantitative RT-PCR was performed with an ABI 7500 Fast System (Applied Biosystems). Comparison of transcript levels was performed by the ΔC_T method of analysis using *tufA* as the endogenous control gene. The Taqman primers and probes used in this study are listed in Supplementary Table S2.

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