Collagen VI Encodes Antimicrobial Activity: Novel Innate Host Defense Properties of the Extracellular Matrix

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Key Words

Antimicrobial activity • Innate host defense •
Collagen type VI • Extracellular matrix • Streptococcus •
Oral pathogens

Abstract

Collagen type VI is a subepithelial extracellular matrix component in airways and an adhesive substrate for oral pathogens [Bober et al.: J Innate Immun 2010;2:160–166]. Here, we report that collagen VI displays a dose-dependent antimicrobial activity against group A, C, and G streptococci by membrane disruption in physiological conditions. The data disclose previously unrecognized aspects of the extracellular matrix in innate host defense.

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Introduction

Collagen type VI is a ubiquitous fibrillar component of the mammalian extracellular matrix [for references, see 1–3]. It is present in most connective tissues, often associated with basement membranes. The collagen VI subunits are heterotrimers composed of $\alpha 1$, $\alpha 2$, and $\alpha 3$ polypeptide chains, where additional tissue-specific chains ($\alpha 4$, $\alpha 5$, and $\alpha 6$) may substitute for the $\alpha 3$ chain in

some situations [2, 4]. Structurally the α -chains are organized as dumb-bell-shaped monomers, where a short extended triple-helical region is flanked by two large N- and C-terminal globular regions. Four monomers align to tetramers by lateral association, which then aggregate end-on-end to microfibrils that become part of extended supramolecular matrix assemblies. The N- and C-terminal globular domains share homology with von Willebrand factor type A domains [5]. Interestingly, von Willebrand factor harbors cationic sequence motifs associated with heparin affinity that may confer antimicrobial properties [6]. Similar motifs are contained in the collagen VI sequence [5] and are conserved between different species.

Group A streptococcus is an exclusive human pathogen that preferentially colonizes pharyngeal epithelium, skin, and soft tissues. Infections may cause pneumonia and serious invasive conditions like septicemia, toxic shock syndrome, and necrotizing fasciitis [recently reviewed in 7]. Group C and G streptococci are commonly isolated as commensals from upper airways, skin, and the gastrointestinal tract, but they are also associated with infections of serious clinical importance. They may cause a spectrum of diseases similar to those caused by group A streptococci, from pharyngitis and impetigo to severe conditions such as necrotizing fasciitis [for references, see 8]. Pharyngeal carriage of these bacteria may be an underlying cause of acute rheumatic fever [9].

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Primary adhesion to host targets is generally considered to be crucial in bacterial pathogenesis [10], enabling the microbe to successfully invade and colonize the host and bypass host defense mechanisms [11]. In addition to cell surface receptors, extracellular matrix components such as collagen I and IV, fibronectin, laminin, and vitronectin have been reported to be microbial target structures [for references, see 11–13]. Recently, we identified collagen VI as an adhesive substrate for oral pathogens *Streptococcus pyogenes* and *Streptococcus pneumoniae*. M1 protein was identified as a collagen VI binding adhesin of *S. pyogenes* [1].

During these studies, given the fact that the collagen VI sequence contains cationic motifs with antimicrobial potential, we were prompted to ask whether this intriguing multidomain molecule exerts antibacterial activity. Indeed, our data disclose a previously unrecognized antimicrobial potential of collagen type VI against oral pathogens. Killing of A, C, and G streptococci occurs in a dose-dependent way by membrane permeabilization in physiological conditions. As a consequence, extracellular matrix components may protect from pathogen invaders by aiding innate host defense responses that are beneficial in early stages of infection.

Materials and Methods

Microorganisms and Culture Conditions

The following bacterial strains were kindly provided by Inga-Maria Frick (Department of Infection Medicine, Lund University): group A streptococcus AP1 (strain 40/58 of the M1 serotype) and isogenic mutants lacking protein M1 (BMJ11), protein H (BMJ27.6), or both M1 and H (BMJ71) [14]; group C streptococcus C16 and C36 [15, 17], and group G streptococcus G41 and G148 [16, 17]. All strains were grown overnight in Todd-Hewitt broth (THB; Gibco, Grand Island, N.Y., USA) at 37°C in a humid atmosphere containing 5% CO₂. BMJ11 and BMJ71 were supplemented with tetracycline (5 µg/ml), and for BMJ27.6, erythromycin (1 µg/ml) was added to the THB medium.

Isolation of Type VI Collagen

Collagen type VI microfibrils were extracted from bovine cornea by collagenase digestion as described by Spissinger and Engel [18] with some modifications. Calf eyes were obtained from the local abattoir. Corneas were dissected and cut into pieces in digestion buffer: 50 mM Tris-HCl, 150 mM NaCl, and 5 mM CaCl₂ (pH 7.4), supplemented with proteinase inhibitors: 10 mM N-ethylmaleimide, 2 mM phenylmethanesulfonylfluoride, 10 mM benzamidine hydrochloride, and 100 mM aminocaproic acid. The corneas were homogenized in digestion buffer using a Polytron homogenizer (Kinematica AG, Littau, Switzerland), followed by digestion with 20 mg bacterial collagenase IA (197 U/mg; Worthington, N.J., USA) per gram of wet tissue in digestion buffer overnight at

room temperature under gentle stirring. The digest was centrifuged at 32,000 g for 30 min at 4°C and the supernatant was applied to a Sepharose CL-2B column (Pharmacia, Uppsala, Sweden) in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The column (column volume 2 liters) was run at 2 ml/min and 10-ml fractions were collected. The protein content was determined at 280 nm in a spectrophotometer and protein-containing void volume fractions were examined by electron microscopy. The purity was confirmed by mass spectral analysis (MALDI-TOF Voyager; Applied Biosystems, Foster City, Calif., USA). The collagen type VI-containing samples were stored at 4°C until further use.

Antibacterial Activity Assay

Bacteria were grown to the mid logarithmic phase in Todd-Hewitt broth (OD₆₂₀ ≈ 0.4), harvested by centrifugation at 3,500 rpm for 10 min, and washed twice in TBS buffer. Bacterial suspensions were adjusted to 2 × 10⁹ colony-forming units (cfu) per ml. The bacteria were further diluted in TBS and incubated with different collagen VI concentrations (10 nM, 100 nM, or 2 µM, respectively). Bacteria incubated with TBS or 3 µM LL-37 (Innovagen, Lund, Sweden) were used as controls. Samples were incubated for 2 h at 37°C in a humid atmosphere containing 5% CO₂. Serial dilutions were plated on blood agar plates and incubated overnight at 37°C, 5% CO₂, and the number of cfu was thereafter determined by counting visible colonies. Experiments were performed in triplicate.

Transmission Electron Microscopy

For negative staining and transmission electron microscopy, samples were adsorbed onto 400 mesh carbon-coated copper grids and stained with 0.75% (w/v) uranyl formate as recently described in detail [19]. Samples were observed in an FEI Tecnai™ G2 Spirit (North America NanoPort, Hillsboro, Oreg., USA) transmission electron microscope operated at 60 kV accelerating voltage. Images were recorded with an Olympus SIS Veleta CCD camera.

Results

Collagen Type VI Is Antimicrobial against *S. pyogenes*

In order to assess possible antibacterial effects of collagen type VI, we treated *S. pyogenes* with purified preparations of this protein. Bacteria treated with TBS buffer or the cathelicidin peptide LL-37 [20] served as negative and positive controls, respectively. The results from viable-count assays show dose-dependent killing of AP1 (fig. 1a). Treatment with collagen VI for 2 h at 37°C significantly inhibited the growth of *S. pyogenes* as compared to control bacteria treated with TBS. Different collagen VI concentrations between 10 nM and 2 µM were applied. Notably, at micromolar collagen concentrations the efficiency of bacterial clearance was comparable to the 'classical' human antimicrobial peptide LL-37.

Collagen VI Killing Properties Are Associated with Streptococcal Membrane Disruption

For a more detailed understanding of the underlying killing mechanism, streptococci were incubated with 1 μM collagen VI and visualized by negative staining and electron microscopy. Figure 1b–e depicts the sequence of events leading to bacterial inactivation. Initially, collagen VI microfibrils adhere to the streptococcal surface (fig. 1b) as reported previously [1]. The globular von Willebrand A domains of the collagen are frequently seen in close contact with surface structures (fig. 1b, c, asterisks). Subsequent to this initial step, membrane perturbations, blebbing (fig. 1c, arrowheads), and exudation of cytoplasmic content (fig. 1c, arrows) are observed. Large scale membrane destabilization events (fig. 1d, arrowhead) finally lead to disintegration of the bacterial cells into a mixture of membrane vesicles and cytoplasmic ejecta (fig. 1e). Taken together, the data presented in figure 1 show that collagen type VI exerts antimicrobial activity against AP1 by membrane rupture. The effect is dose dependent at physiological pH and salt concentrations.

Killing of Group A Streptococci Correlates to M1 Surface Protein Expression

Adherence to the bacterial surface and interaction with the membrane are a prerequisite for the antimicrobial action of a given antimicrobial agent. We have recently shown that on the *S. pyogenes* surface protein M1 is an adhesin for collagen VI. Another important virulence factor of *S. pyogenes*, protein H, does not exhibit affinity for this collagen [1]. In order to delineate the antimicrobial properties of collagen VI in further detail, we analyzed isogenic mutant strains of *S. pyogenes* expressing or lacking these surface proteins and thus the ability to bind collagen VI. Wild-type AP1 bacteria and BMJ11 (M1 deficient), BMJ27.6 (protein H deficient), and BMJ71 (M1 and H deficient) [14] were used in antimicrobial assays as described above. Indeed, streptococci lacking M1 protein, i.e. BMJ11 and BMJ71, showed a considerably higher viability than AP1 or the protein H mutant BMJ27.6 (fig. 2a). These results were confirmed by negative staining and transmission electron microscopy (fig. 2b). The M1 protein-expressing strains AP1 and BMJ27.6 were more affected by membrane rupture and cytoplasmic exudation than the M1 mutants. Although BMJ11 and BMJ71 were somewhat affected as compared to control bacteria, they were less prone to killing by collagen VI. These findings suggest a crucial role for the interaction between collagen VI and M1 protein during the elimination of A streptococcus.

Fig. 1. Collagen type VI microfibrils induce killing of group A streptococcus by membrane destabilization. Antibacterial activity assay with *S. pyogenes* and collagen VI. AP1 bacteria were incubated with different concentrations of collagen VI for 2 h at 37°C. Bacteria incubated with TBS buffer or with LL-37 served as negative or positive controls, respectively (a). Molar concentrations are indicated in the figure. The dots represent individual experiments with the respective strain. **b–d** Negative staining and transmission electron microscopy of streptococcal membrane destabilization upon incubation with collagen VI. Initially, collagen microfibrils assemble at the bacterial surface (b). The globular von Willebrand A domains of collagen VI are often observed close to the bacterial surface (asterisks). Subsequently, membrane blebbing (c, arrowheads) and exudation of cytoplasmic content are visible (c, arrows). Large scale membrane disruption (d) finally leads to destruction of the bacteria cells (e). Scale bar = 100 nm.

Fig. 2. Correlation between bacterial killing efficiency and expression of M1 surface protein. **a** Incubation of *S. pyogenes* wild-type strain AP1 and isogenic mutants BMJ11, BMJ27.6, and BMJ71 with 1 μ M collagen VI, followed by quantification of survival (cfu) in an antibacterial activity assay. In control samples bacteria were incubated with TBS buffer or LL-37. The dots represent individual experiments with the respective strain. **b** Specimens were analyzed by negative staining and electron microscopy. Scale bar = 1 μ m. Mutants lacking M1 protein (BMJ11 and BMJ71) are less prone to growth inhibition (**a**) and membrane destabilization (**b**, arrowheads) than wild-type AP1 and the protein H mutant BMJ27.6.

Collagen VI Is Antimicrobial against Group A, C, and G Streptococci

To examine the antimicrobial properties of collagen VI towards a spectrum of oral pathogens, we tested patient isolates of group C and G streptococci in comparison to *S. pyogenes*. In viable count assays the bacterial growth of C16, C36, G41, and G148 was inhibited significantly, as observed for AP1. The collagen VI killing mechanism by membrane permeabilization was assessed by electron microscopic analysis. In contrast to incubation with TBS alone, all bacteria which were exposed to collagen VI exhibited membrane disruption and leakage of cytoplasmic material (fig. 3). For all examined streptococcal species this effect correlated with the collagen concentration, where micromolar concentrations of collagen VI (fig. 3c) exhibited a higher degree of membrane disruption than nanomolar doses (fig. 3b).

Discussion

This study demonstrates that collagen VI, a ubiquitous fibrillar extracellular matrix component, harbors antimicrobial activity against Gram-positive oral pathogens. Killing of group A, C, and G streptococci is dose dependent and occurs by membrane disruption in physiological conditions. Similar observations with Gram-negative microorganisms from the lower airways are under present investigation [Abdillahi, unpubl. res.]. The data uncover a previously unrecognized role of collagen VI as an innate host defense effector molecule in connective tissues.

The integrity of the airway mucosa is crucial for its protective properties against potentially invasive oral pathogens and diseases transmitted through the respiratory tract. When the epithelial cell layer is damaged by postviral infection injury or epithelial shedding, the underlying submucosa becomes exposed and vulnerable to pathogen invaders. In this scenario a repertoire of antimicrobial activity is beneficial to reduce the susceptibility of the tissue for infection. Indeed, during the past years evidence has emerged that connective tissues possess antimicrobial peptides related to extracellular matrix molecules as part of the natural tissue immune response [6, 21–23]. Our data indicate that subepithelial collagen VI scaffolds, encoding both adhesive and antimicrobial properties, provide environments that may enhance innate antimicrobial protection in the respiratory tract.

Fig. 3. Dose-dependent killing of group A, C, and G streptococci by collagen VI. Electron microscopy of different streptococcus species after incubation with TBS (upper panel), 10 nM collagen VI (middle panel), or 2 μ M collagen VI (lower panel). Group A streptococci (GAS), group C streptococci (GCS), and group G streptococci (GGS) are indicated in the figure. All different streptococci are killed by collagen VI in a dose-dependent manner by membrane permeabilization and cytoplasmic exudation. Scale bar = 1 μ m.

The killing efficiency of group A streptococcus was modulated by M1 protein, a recently identified collagen VI adhesin. In the absence of M1 on the streptococcal surface, killing was considerably less pronounced, indicating that the binding of collagen VI close to the bacterial membrane is crucial for its membrane-disrupting ability. The antibacterial effect was not completely abolished, though, suggesting that other still unrecognized collagen-binding surface structures might exist and promote some killing. These observations point to an inherent complex role of M1 protein in host-parasite interplay during infection. On the one hand, as a collagen VI adhesin, M1 is a streptococcal virulence factor facilitating host engagement and successful colonization. On the other hand, M1 can contribute to a microenvironment discouraging bacterial growth by targeting antimicrobial sites in the collagen VI molecule. This spectrum of innate adhesive and protective properties may cause different net effects in different scenarios of infection, highlighting the delicate balance in the host-microorganism relationship.

Electron microscopy showed that collagen VI induces a membrane permeabilization effect similar to the 'classical' antimicrobial cathelicidin peptide LL-37 [24–26]. Our data do not, however, demonstrate the exact killing mechanism by collagen VI because secondary metabolic effects may trigger bacterial death and membrane disruption. Even so, it is an intriguing hypothesis that col-

lagen VI may directly target the bacterial membrane with its von Willebrand A domains. Subsequently, antimicrobial peptides in the A domains could inactivate the microbe by membrane destabilization, either directly within the A domains or released by bacterial or inflammatory proteases. In future work it will be important to identify and characterize such antimicrobial motifs in the collagen VI molecule.

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