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Human colonic mucus is a reservoir for antimicrobial peptides☆

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Abstract

Background and aims: To prevent bacterial adherence and translocation, the colonic mucosa is covered by a protecting mucus layer and the epithelium synthesizes antimicrobial peptides. The present qualitative study investigated the contents and interaction of these peptides in and with rectal mucus.
Methods: Rectal mucus extracts were analyzed for antimicrobial activity and screened with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Dot blot and immunohistochemistry for antimicrobial peptides. In addition, binding of AMPs to mucins was investigated by Western blot and enzyme-linked lectin assays.
Results: In functional tests the mucus layer exhibited a strong antimicrobial activity. We detected 11 antimicrobial peptides in mucus extracts from healthy persons including the defensins HBD-1 and -3, the cathelicidin LL-37, ubiquitin, lysozyme, histones, high mobility group nucleosome-binding domain-containing protein 2, ubiquicidin and other ribosomal proteins. AMPs were bound by mucins but this was demonstrated to be reversible and inhibition of antibacterial activity was limited.
Conclusion: These findings indicate that epithelial antimicrobial peptides are retained in the intestinal mucus layer without losing their efficacy. Thus, the mucus layer and its composition provide an attractive drug target to restore antimicrobial barrier function in intestinal diseases.

Abbreviations: AMP, antimicrobial peptide; ELLA, enzyme-linked lectin assay; HMGN2, high mobility group nucleosome-binding domain-containing protein 2; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography.

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1. Introduction

The human gastrointestinal tract is colonized by a variety of different microorganisms.[1] Containing 10^{11-12} bacteria per gram of feces, especially the colon is confronted with a massive bacterial load.[2] To prevent bacterial adherence and translocation, the mucosa is protected against this potentially harmful environment by an efficient physical and chemical barrier. This complex defense system comprises numerous innate protective mechanisms, including the production of a large variety of antimicrobial peptides (AMPs) and the intestinal mucus layer.[1,3] AMPs are used as a first line of immune defense by many organisms including plants, insects, bacteria and vertebrates.[4] Collectively, their antimicrobial spectrum reaches from killing of bacteria and some parasites to antifungal and antiviral activities.[5] The two most important families of AMPs in mammals are the defensins and the cathelicidins.[6]

Human defensins are subdivided into two groups, α- and β-defensins, that differ in the length of the peptide segments between 6 conserved cysteine residues and their disulfide-bonding pattern.[2] They are small cationic peptides with a molecular weight of 3–5 kDa.[7] Defensins are either produced constitutively, like the human β-defensin-1 (HBD-1) and the Paneth cell α-defensins, or they are inducible by proinflammatory stimuli and microbes or their products, like the human β-defensins HBD-2 and HBD-3.[8–12]

The only cathelicidin identified in humans is called LL-37/hCAP-18.[6] It is expressed by neutrophils, keratinocytes and epithelial cells including the colon and possesses a broad spectrum of activity against Gram-positive bacteria, Gram-negative bacteria and *Candida albicans*.[2] Other proteins with reported antibacterial activity are histones, ribosomal proteins and enzymes like lysozyme and phospholipase A_2.[13,14] In the human colon, a complex mixture of antimicrobial peptides is involved in the mucosal defense. In addition to the constitutively expressed β-defensin HBD-1, the cathelicidin LL-37, the inducible β-defensins HBD2–4, and the α-defensins HNP1–3, antimicrobially active members of the histone family, phospholipase A_2, an eosinophil cationic protein and the ribosomal proteins L30, L39, S19 and S30 (ubiquicidin) have been already identified in the colonic mucosa.[15–18] In addition to these directly active antimicrobial defense mechanisms, the intestinal mucosa is also protected by the intestinal mucus layer. This layer is a hydrated polymeric gel covering the mucosal surface. It protects the epithelium against mechanic disruption, toxins, enzymes and microorganisms and also has a lubricating function facilitating the passage of luminal contents.[19,20]

The colonic mucus layer consists of two parts — a normally sterile inner layer firmly attached to the epithelium and a loosely adherent outer layer colonized by bacteria.[21] Large glycoproteins belonging to the family of the secreted mucins are the major structural components of this protective gel. In the human gastrointestinal tract and associated tissues, the gel-forming secreted mucins MUC2, MUC5AC, MUC5B, MUC6 and MUC19 are expressed.[22] MUC2 is the predominant gel-forming mucin in the human colon.[3,23] Its major importance for the maintenance of an intact mucus layer has been shown in $MUC2^{-/-}$ mice that spontaneously developed colitis.[24] The carbohydrate structures of colonic mucins carry sulfate- and sialic acid residues, giving the mucins a highly negative charge.[25,26] Owing to this negative charge and the viscoelastic net-like composition of mucus, the cationic positively charged AMPs might be retained in the large intestinal mucus layer. In the mouse small intestine it was recently suggested that indeed the secreted antimicrobial activity localizes to the mucus surface layer.[27] In this study, we tested the hypothesis of the mucus layer as a reservoir for antimicrobial components in the human large intestine.

2. Material and methods

2.1. Patients and sample preparation

Rectal mucus samples were collected during routine endoscopies at the Robert-Bosch-Hospital (Stuttgart) using sterile cytology brushes. Patients suffering from inflammatory bowel diseases or other rectal pathology were excluded. In addition, surgical resections of colonic tissue from patients with colon carcinoma were obtained from the Department of Surgery at our hospital.

Proteins from mucus samples were extracted by incubation in 60% acetonitrile containing 1% trifluoroacetic acid (TFA) according to Meyer-Hoffert et al.[27] with slight modifications. Subsequently, samples were centrifuged for 10 min at 8000 *g* and the supernatants were lyophilized. The pellets were re-dissolved in 10% ethanol and protease inhibitors were added. For subsequent experiments either this total protein extract was used or it was further fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) on an Agilent 1200 series system using a Zorbax 300SB-C18 column (4.6 × 150 mm, 3.5 μm, Agilent Technologies, Waldbronn, Germany) and a water/acetonitrile gradient (solvent A: 0.18% TFA/H_20, solvent B: 0.15% TFA/acetonitrile). Fractions of 1.5 ml were collected, lyophilized and re-suspended in 0.01% acetic acid.

2.2. Antimicrobial assays

The antimicrobial activity of total mucus extracts from 6 control persons was assessed using a flow cytometric assay.[28] Briefly, suspensions of *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacteroides fragilis* ATCC 25285 and the clinical isolate *Candida albicans* 526 were grown in Schaedler Broth (diluted 1:6 with sterile water) at 37 °C over night, *B. fragilis* in an anaerobic jar with Anaero Gen (Oxoid, Wesel, Germany). Subsequently, 1.5×10^6 bacteria/ml in 1:6 diluted Schaedler Broth were incubated with 50 μg total protein of each total mucus extract in a final volume of 100 μl for 120 min at 37 °C. Bacteria incubated with 5 μl 0.01% acetic acid and 5 μg/100 μl recombinant HBD-3 (PeptaNova, Sandhausen, Germany) served as negative and positive controls respectively. Afterwards, the samples were incubated with 1 μg/ml bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Invitrogen/Life Technologies).

For investigation of HPLC fractionated extracts *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were incubated with 6 μl extract as described above.