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A flow cytometric assay to monitor antimicrobial activity of defensins and cationic tissue extracts

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Abstract

To determine the antibacterial activity of defensins and other antimicrobial peptides in biopsy extracts, we evaluated a flow cytometric method with the membrane potential sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$]. This assay enables us to discriminate intact non-fluorescent and depolarized fluorescent bacteria after exposure to antimicrobial peptides by measurement at the direct target, the cytoplasmic membrane and the membrane potential. The feasibility of the flow cytometric assay was evaluated with recombinant human β-defensin 3 (HBD-3) against 25 bacterial strains representing 12 species. HBD-3 showed a broad-spectrum dose dependent activity and the minimal dose to cause depolarization ranged from 1.25 to >15 μg/ml HBD-3, depending on the species tested. The antibacterial effect was diminished with sodium chloride or dithiothreitol and could be abrogated with a HBD-3 antibody. Additionally, isolated cationic extracts from human intestinal biopsies showed a strong bactericidal effect against *Escherichia coli* K12, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, which was diminished towards *E. coli* at 150 mM NaCl, whereas the activity towards *S. aureus* ATCC 25923 remained unaffected at physiological salt concentrations. DTT blocked the bactericidal effect of biopsy extracts completely.

Keywords: Antimicrobial activity; Bacteria; $DiBAC_4(3)$; Flow cytometry; HBD-3

1. Introduction

The synthesis of antimicrobial peptides is known as a common mechanism in innate epithelial defense against invading microorganisms by a multitude of species from plants, lower and higher vertebrates to humans (Zasloff, 2002). Major representatives of this barrier are the ubiquitous defensins, which play an important role in innate immunity and inhibit colonization of the epithelium by pathogenic microorganisms (Ouellette, 1999). Defensins are cationic and hydrophobic peptides with a molecular mass ranging from 3 to 6 kDa. They exhibit a broad-spectrum antimicrobial activity against bacteria, fungi and some enveloped viruses and can also act as chemokines (Chertov et

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al., 1996; Yang et al., 1999). Based on the position of 3 intramolecular disulfide bridges defensins are classified as α- or β-defensins (Nissen-Meyer and Nes, 1997). Up to now 6 α-defensins (HNP1-4, HD-5 and HD-6) and 6 β-defensins (HBD-1 to-6) have been identified in humans. Defensins are present in neutrophils and on nearly all epithelial surfaces of the human body (Bensch et al., 1995; Ganz et al., 1985; Garcia et al., 2001a,b; Harder et al., 1997, 2001; Yamaguchi et al., 2002). Their mechanism of action is characterized as attachment of the cationic defensins to the negatively charged bacterial cell surface resulting in electrostatic charge-based membrane permeabilization or in pore formation followed by a membranolytic disruption of the plasma membrane (Harder et al., 2001; Kagan et al., 1990; Shimoda et al., 1995).

HBD-3 has a molecular mass of 5.16 kDa, exhibits a broad-spectrum antimicrobial activity (Maisetta et al., 2003) and was originally isolated from human lesional psoriatic scales (Harder et al., 1997). HBD-3 is produced by various epithelial and non-epithelial tissues (Nishimura et al., 2003), Garcia et al. (2001a) found a baseline expression of HBD-3 in leukocytes, placenta, testis, heart and skeletal muscle and Fahlgren et al. (2004) detected HBD-3 in the intestinal epithelium.

The intestinal mucosa synthesizes in addition to defensins other broad-spectrum antimicrobial peptides as for example lysozyme, lactoferrin, cathelicidins, phospholipase A, histones and ribosomal proteins (Cunliffe and Mahida, 2004; Howell et al., 2003; Tollin et al., 2003). Prior data of our working group based on real time RT-PCR and immunohistochemistry showed that the intestinal pattern of defensin expression is different in Crohn's disease and ulcerative colitis compared with patients without inflammatory bowel disease (Wehkamp et al., 2002b; Wehkamp et al., 2002a). These findings suggest that an altered mucosal defensin expression may allow bacterial overgrowth, adherence and invasion and plays an essential role in the development of inflammatory bowel disease (Fellermann et al., 2003). To elucidate the functional consequences of a modified defensin expression in the intestinal mucosa of patients with IBD our working group investigates the antimicrobial effect of protein extracts from intestinal biopsies from patients with Crohn's disease, ulcerative colitis and controls. Therefore, in this study we employed an assay to examine the antimicrobial activity of cationic peptides extracted from mucosal biopsies. This method for determination of cell viability was recently established primarily for antibiotic susceptibility testing of bacteria in a clinical routine setting (Nuding and Mueller, 2004). The discrimination between intact and damaged bacterial cells is based on flow cytometry in conjunction with a membrane potential sensitive fluoroprobe. Cell wall damage or cell death cause a depolarisation of the membrane potential, which can be detected with membrane potential sensitive dyes (Jepras et al., 1997). The anionic dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] has only a low binding affinity for intact membranes and is limited to the outer regions of the cell membrane in living bacteria. Depolarization leads to an uptake of dye inside the cell according to the Nernst equation and the dye binds to lipid-rich compounds within the cell resulting in an increasing fluorescence signal detected by flow cytometry (Deere et al., 1995). Hence, intact non-fluorescent and damaged fluorescent bacteria of a population can well be differentiated. The great advantage of this flow cytometric test is the possibility to quantitate the antimicrobial effect of defensins at the direct targets, the cell membrane and the membrane potential.

To evaluate the flow cytometric assay for investigation of the antimicrobial activity of protein extracts from biopsies, we first examined the effect of HBD-3 against different bacterial strains, including Gram-negative and Gram-positive bacteria, flagellated and capsulated species as well as anaerobic species.

After validation of the method with HBD-3, we adopted the technique to mucosal extracts. Whole protein extracts and cationic fractions from intestinal biopsies were tested for their bactericidal activity. The sensitivity towards sodium chloride and dithiotreitol, which can inactivate defensins, was assessed for further characterization.

2. Materials and methods

2.1. Bacterial strains and bacterial growth

The tested representative bacterial spectrum comprised a total of 25 strains, including reference strains from the American Type Culture Collection (ATCC) and clinical isolates of *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus parasanguis*, *Enterococcus faecalis*, *Bacteroides fragilis* and *Bacteroides vulgatus*.

Initial experiments with different culture media showed that the ionic strength was a major determinant for the antibacterial effect of HBD-3 with higher sodium chloride concentrations inhibiting bacterial killing. In undiluted Luria Bertani Broth or Schaedler Broth the