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Molecular Immunology 42 (2005) 575–579
Molecular Immunology
www.elsevier.com/locate/molimm

Detection of individual human neutrophil α-defensins (human neutrophil peptides 1, 2 and 3) in unfractionated gingival crevicular fluid—A MALDI-MS approach

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Received 20 July 2004
Available online 10 November 2004

Abstract

The role of antimicrobial peptides is particularly important in the oral cavity where there is constant challenge by microorganisms. The α-defensins are a group of cationic peptides that comprise 30–50% of the total protein in azurophilic granules of human neutrophils. They include the human neutrophil peptides (HNP) 1, 2 and 3 which have almost identical amino acid sequences but differ in their biological activities. The amino acid sequence similarities of the defensins have made it difficult to unequivocally determine the presence of individual defensins using antibody-based techniques. However, by virtue of their cationic nature we postulated that the defensins would fly particularly well in mass spectrometry and that this characteristic would allow facile identification of individual HNPs in unfractionated gingival crevicular fluid (GCF) from periodontitis patients and healthy controls. Although there was variability in levels of defensins detected in periodontal health and disease, HNP-1 was always identified as the major peak in the triad and HNP-3 as the minor peak, lending support to the hypothesis that HNP-2 may arise by post-translational proteoyltic cleavage of HNP-3 rather than HNP-1. The finding that the defensins were more abundant in a higher proportion of the healthy sites studied could be linked to a more intact defensin barrier in periodontal health.
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Keywords: Defensin; GCF; MALDI-MS; Periodontitis

1. Introduction

Periodontal inflammation represents the host response to bacterial plaque, mediated by the environment in which the response occurs. As periodontitis progresses, the supporting tissues of the teeth, including the periodontal ligament and alveolar bone, are destroyed, ultimately leading to tooth loss in severe cases (Genco, 1990). Although it is well recognised that the primary etiological factor in periodontal disease is the presence of gram-negative bacteria, the importance of host susceptibility to disease is receiving much deserved attention. Indeed it is recognised that the host response to infection plays a major role in controlling the tissue destruction observed in periodontitis (Van Dyke and Serhan, 2003).

The recruitment of polymorphonuclear leukocytes (PMNs) and other inflammatory cells to the periodontal pocket is an important feature of the inflammatory process in periodontal disease. PMNs are the first cells to arrive in response to an antigenic challenge and are key effector cells in early inflammation. Despite being predominantly defensive, PMNs can also have pro-inflammatory effects as a result of their interactions in the periodontal pocket. In periodontal health and disease, neutrophils play a critical role in defending the host against infection by ingesting and killing invading microorganisms. Neutrophils contain an abundance of antimicrobial peptides which contribute to the oxygen-independent killing of microorganisms in the extracellular

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doi:10.1016/j.molimm.2004.09.008

environment. The most abundant of these antimicrobial peptides are the so-called 'defensins'.

The defensin family of antimicrobial peptides are widely distributed among mammals insects and plants and have an important role in the innate immune response (Ganz, 2003). The α- or classical defensins, the first group to be isolated from human and animal phagocytes, comprise a large subfamily of polypeptides containing 29–40 residues (Lehrer et al., 1993). They are cationic peptides characterised by eight invariant residues within their amino acid sequence, including six cysteines. Humans produce at least six different α-defensins including four human neutrophil peptides (HNP) numbered 1–4. HNP-1, HNP-2 and HNP-3 are the most abundant, comprising 30–50% of the total protein in the azurophilic granules of human neutrophils. The remaining α-defensins, known as human defensins 5 and 6 (HD-5, HD-6), are localised in the secretory granules in the Paneth cells that are situated in the crypts of the small intestine. The phylogenically older human β-defensins are more basic, slightly longer peptides that are expressed in various mucosae and epithelial cells (Krisanaprakornkit et al., 1998). Although the α- and β-defensin families do not share DNA sequence similarity or disulphide topology, they possess highly similar tertiary structures and exhibit common properties such as antimicrobial activity.

HNP-1, HNP-2 and HNP-3 have almost identical amino acid sequences (Lehrer et al., 1993). HNPs 1 and 3, which are both 30 amino acid peptides, are encoded by similar genes on chromosome 8. They differ only in their N-terminal amino acid, which is alanine in HNP-1 but is substituted by aspartate in HNP-3. It is believed that HNP-2, which is 29 amino acids in length, could arise by post-translational proteolytic cleavage of the N-terminal amino acid of HNP-1 and/or HNP-3 (Fig. 1).

Although HNPs 1–3 have almost identical sequence homology, they have markedly different biological properties. For example, HNP-1 has potent antifungal activity against *Candida albicans*, whereas the activity of HNP-3 is minimal (Lehrer et al., 1988). This divergence in biological properties warrants further study to determine whether variations in the levels of HNPs 1–3 are associated with inflammatory diseases such as periodontitis. Because of their amino acid sequence similarities, antibodies to one human neutrophil defensin will invariably cross react with all other HNPs. Therefore, it has not been possible to unequivocally identify each of the individual HNPs within an inflammatory milieu because of their structural similarities. Analysis of the presence of individual α-defensins is further complicated by the fact that studies of mRNA expression could only infer levels of HNPs 1 and 3, since HNP-2 arises post-translationally.

HNP-1: ACYCRIPACIAGERRYGTCIYQGRLWAFCC

HNP-2: CYCRIPACIAGERRYGTCIYQGRLWAFCC

HNP-3: DCYCRIPACIAGERRYGTCIYQGRLWAFCC

Fig. 1. Amino acid sequences of HNP-1, HNP-2 and HNP-3.

By virtue of their cationic nature we postulated that the defensins would fly particularly well in mass spectrometry and that this characteristic would allow facile identification of individual HNPs in an unfractionated inflammatory exudate such as gingival crevicular fluid (GCF). The study of GCF samples from defined sites of chronic periodontal inflammation allows non-invasive access to an inflammatory exudate, which can be used to improve our understanding of the inflammatory process. The aim of the current study was to determine whether HNPs 1–3 could be detected in unfractionated GCF using mass spectrometry and to determine whether there were differences in the HNP profile in periodontal health and disease.

2. Materials and methods

2.1. Patients and samples

2.1.1. Periodontitis subjects

The periodontitis subjects studied were patients referred to the Periodontal Department, School of Dentistry, Queen's University, Belfast for specialist management. The inclusion criteria for this group were the presence of established periodontitis (Machtei et al., 1992) in subjects who had not received any periodontal treatment in the previous six months. The exclusion criteria for this group included diabetes, bleeding disorders, gross oral pathology or treatment in the previous six months with antibiotics or anti-inflammatory drugs. The study was approved by the ethical committee of the Faculty of Medicine, Queen's University, Belfast and informed, written consent was obtained from those who agreed to participate.

In each periodontitis subject one site with periodontitis was identified for sample collection by visual inspection, existing clinical records and radiographs. Criteria for periodontitis were gingival index $\geq$1, probing depth $\geq$5 mm with evidence of bleeding on probing, and radiographic evidence of crestal bone loss. The gingival index and probing depth were measured after GCF collection to confirm the site assessment and avoid contamination of the sample with blood. Probing pocket depths were measured from the gingival margin to the base of the clinical pocket with the probe tip parallel to the long axis of the tooth and positioned interproximally as close as possible to the contact point. Measurements were made to the nearest millimetre and where any doubt existed the lower value was scored. All clinical measurements were recorded by an experienced periodontist using Michigan O periodontal probes with Williams markings.

2.1.2. Control subjects

Subjects included in this group had clinically healthy gingival tissues, no visible plaque and no pocketing >3 mm or loss of clinical attachment >2 mm. One healthy site was se-

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