Histatins: Multifunctional Salivary Antimicrobial Peptides

Wim van 't Hof, Menno J. Oudhoff, and Enno C.I. Veerman

Abstract In this chapter, we overview the plethora of properties that have been attributed to histatins including tannin binding, microbicidal activity, immunomodulatory activity, and the recently found stimulation of cell migration. Attention is in particular paid to the molecular mechanisms underlying these properties. We conclude that many properties of histatins can be explained by their physicochemical properties, which allows them to bind a variety of negatively charged molecules and surfaces. For instance, their cationic character is crucial for their membrane-disrupting activity, which forms the basis of their antimicrobial activity. The only function that cannot directly be predicted on the basis of their physicochemical features is the enhancement of wound healing which proceeds via canonical receptor-mediated cell signalling.

1 Introduction

Histatins were among the first antimicrobial peptides that were discovered (MacKay et al. 1984a, b; Pollock et al. 1984). They make up a family of 12 histidine-rich peptides that occur exclusively in the saliva of humans and higher primates (Sabatini et al. 1989; Xu et al. 1990). They are encoded by two genes: *HTN1* and *HTN2* (also referred to as *HTN3* or *HTN5*). The HIS1(1) allele on *HTN1* encodes histatin 1 and the derived histatin 2; the HIS2(1) allele on *HTN3* encodes histatin 3 (Sabatini and Azen 1989), of which all other histatins are derived, presumably by posttranslational proteolytic cleavage (Vanderspek et al. 1990). Of the histatins from human parotid saliva, 85–90 % consists of the histatins 1, 3, and 5 in ratios of 3:1:3 (corresponding to concentrations around 2½ μM, 1 μM, and 4 μM),

W. van 't Hof • M.J. Oudhoff • E.C.I. Veerman (✉)
Section Oral Biochemistry, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Amsterdam, Netherlands
e-mail: e.veerman@acta.nl

P.S. Hiemstra and S.A.J. Zaat (eds.), *Antimicrobial Peptides and Innate Immunity*, Progress in Inflammation Research, DOI 10.1007/978-3-0348-0541-4_7,
© Springer Basel 2013

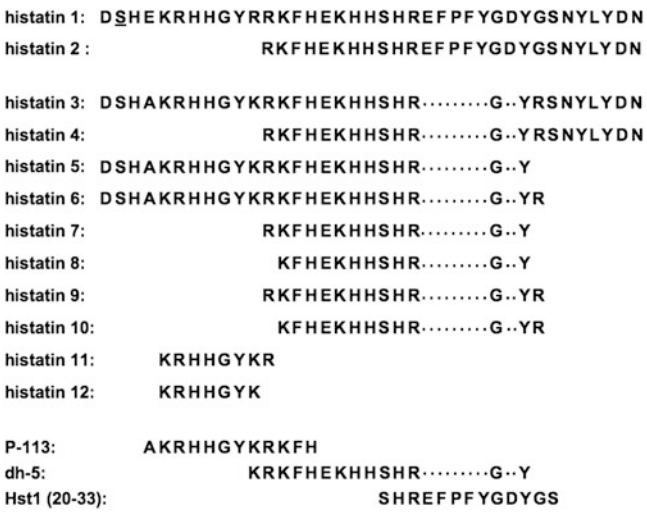

Fig. 1 *Sequence alignment of the histatins and the synthetic variants.* The underscore in the sequence of histatin 1 indicates the presence of a phosphoserine residue at position 2. P-113 and dh-5 are two synthetic minimal-length variants in which the candidacidal potency of the parent peptide, histatin 5, is retained. The large differences between these two peptides, together with the lack of stereospecificity, indicate that the candidacidal activity of histatins is not governed by a proper receptor-binding domain but by a minimal amphipathic stretch. Hst1 (20–33) is the synthetic minimal-length variant in which the epithelial cell-migration-stimulating capacity of the parent peptide, histatin 1, is retained. The corresponding histatin motif is unique for the *HTN1*-derived histatins (1 and 2) and bears neither significant overlap nor structural resemblance with the putative antimicrobial motifs represented by dh-5 and P-113

respectively. Of these, histatin 5 is the most potent peptide, and histatin 1 the least (Oppenheim et al. 1986, 1988). Due to the relatively large contribution of histatin 5, research has primarily focussed on this peptide and its derivatives. Based on activity studies with synthetic truncated variants, two stretches were identified in histatin 5 that can be considered as minimal active domain: residues 11–24 and 4–15, denoted dh-5 (Raj et al. 1990) and P-113 (Rothstein et al. 2001), respectively (Fig. 1).

Over the years, histatins have proved efficient antimicrobial agents in vitro against a broad spectrum of microorganisms. These include Gram-positive bacteria (MacKay et al. 1984a, b), Gram-negative bacteria (Murakami et al. 1992), yeasts and fungi (Brant et al. 1990; Oppenheim et al. 1988; Pollock et al. 1984), and the human protozoan parasite *Leishmania* (Luque-Ortega et al. 2008). In addition, it has become clear that histatins are versatile molecules that exhibit a broad range of properties. In this chapter, we review a number of these properties in the context of human innate immunity.

2 Histatins as Membrane-Disrupting Peptides: Mechanism of Action

The generally accepted cellular target of most antimicrobial peptides is the cytoplasmic membrane. The mechanism of action includes electrostatic attraction of the positively charged peptide to the negatively charged microbial membrane and adoption of an amphipathic peptide structure interacting at the hydrophobic/hydrophilic membrane environment interface, followed by destabilisation and disruption of the membrane leading to lethal efflux of vital cell constituents (Fig. 2). Based on this sequence of events, several mechanistic models have been described, mainly differing from each other in the description of the peptide-induced membrane effects (Van 't Hof et al. 2001). The essential requirement for an antimicrobial peptide is the possession of a positively charged domain that is able to adopt an amphipathic secondary structure. Histatin 5 does not seem to contain a distinct active domain, the fact that dh5 and P-113 are equally active, whereas their sequences only moderately overlap, points to a minimum length of an amphipathic stretch with two positively charged termini (the so-called double wing motif), rather than to a discrete antimicrobial domain. Another observation that supports this non-specific mechanism is that the all-D enantiomers of histatin 5 and P-113 show the same antimicrobial activity as the all-L enantiomers (Rothstein et al. 2001; Ruissen et al. 2003).

Originally, disruption of the microbial membrane was considered as the molecular basis of both their antibacterial (MacKay et al. 1984a) and candidacidal activity (Pollock et al. 1984). However, in the killing of *Candida albicans,* histatins behave differently than canonical antimicrobial peptides. Most salient aspect is that killing of *C. albicans* by histatins proceeds at a much slower rate. Whereas addition of a lethal dose of other antimicrobial peptides almost instantaneously results in complete killing of *C. albicans*, killing by histatins takes 15–60 min to reach completion. Such a slow killing rate was considered incompatible with a membranolytic mechanism of action (Edgerton et al. 1998, 2000; Helmerhorst et al. 2001; Koshlukova et al. 1999; Tsai et al. 1996; Xu et al. 1999). Another argument came from model studies with liposomes as mimics of microbial membranes, showing that histatins showed little, if any, membranolytic activity (Den Hertog et al. 2004; Edgerton et al. 1998; Raj et al. 1994). The putative underlying cause was their poor propensity to adopt an amphipathic conformation. On the one hand, it was reported that histatins do not adopt an amphipathic α-helix (Brewer and Lajoie 2002); on the other hand, it was reported that although histatin 5 and its truncated variants histatin 5(9–24) and histatin 5(11–24) (dh-5, see Fig. 1) were able to adopt α-helical conformations in membrane-mimicking solvents, the resulting hydrophobic moments, a measure of the propensity to adopt an amphipathic structure, were much lower than those of other antimicrobial peptides (Helmerhorst et al. 2001; Raj et al. 1990, 1994, 1998). However, antimicrobial peptides with small hydrophobic moments that display a "conventional" rapid killing are not that unusual (Van 't Hof et al. 2001). Neither does

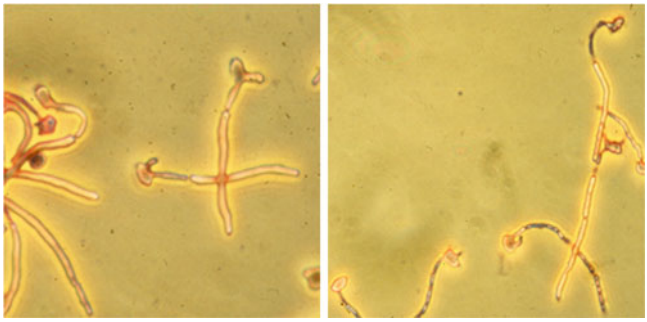

Fig. 2 *Membranolytic effect of histatin 5 on hyphenated C. albicans cells*. Under the microscope, the morphological changes in hyphenated *C. albicans* cells caused by histatin 5 are apparent. In particular, the hyphens appear to have lost their membrane integrity, leading to loss of cell content (*right panel*), compared to untreated viable cells (*left panel*)

the prerequisite of intracellular uptake exclude a membranolytic killing mechanism (Den Hartog et al. 2005; Ruissen et al. 2001).

To evaluate whether histatin 5 acts as a slow pore-former in *C. albicans* cells, we have measured time-dependent membrane depolarisation and permeabilisation as a measure for membrane disruption and correlated these with the degree of cell killing. Contrary to what might be expected from the liposome studies, incubation of *C. albicans* with histatin 5 led to depolarisation of the plasma membrane, as measured using the potential-dependent fluorescent probe $DiSC_3(5)$ (Ruissen et al. 2001). Membrane permeabilisation was monitored using the membrane-integrity probe propidium iodide as well as by measuring the efflux of vital cell components, viz. nucleotides (Den Hertog et al. 2006). Both processes advanced in complete synchronicity with the killing, suggesting an unambiguous relationship between histatin-mediated killing of *C. albicans* and the loss of vital cellular components.

Still, the seemingly absence of membrane disruption by histatins has led to many speculations about alternative mechanisms of action, including energy-dependent receptor-mediated uptake and intracellular targeting (reviewed in: Caldéron-Santiago and Luque de Castro 2009; Isola et al. 2007; Kavanagh and Dowd 2004). Most of these have been proven incorrect (Den Hertog et al. 2006; Isola et al. 2007; Jang et al. 2010; Koshlukova et al. 1999; Ruissen et al. 2001; Veerman et al. 2004, 2007).

In fact, the intracellular uptake of histatins by *C. albicans*, leading to lethal efflux of vital cell components, proceeds more complicated. At lower concentrations, histatin 5 is internalised by receptor-mediated endocytosis and stored in the vacuoles without having any effect on the membrane integrity and viability of the cells (Mochon and Liu 2008). At higher concentrations, histatin 5 crosses the membrane through a mechanism that is dependent on its high cationic charge. Cell sorting experiments demonstrated that the cells with only vacuolar localisation of histatin 5 survived, while none of the cells with cytoplasmic histatin 5 formed colonies. Time-lapse microscopy revealed that histatin 5 induced a single spatially restricted perturbation in the cytoplasmic membrane, which was permeable for small compounds, including histatin 5, propidiumiodide and rhodamine B.

This was accompanied by rapid expansion of the vacuole, by loss of cell volume, and by a rapid efflux of ATP and K^+. The percentages of released ATP and K^+ were proportional to the percentage of killed cells at any time point of the killing curve.

The tendency to form a relatively long-living cluster on the cytoplasmic membrane is a unique feature of histatins that explains the relatively slow killing kinetics. Interestingly, higher salt concentrations do not abrogate the initial interaction of histatins with the cell but arrest the killing process at the formation of these membrane-associated histatin clusters (Mochon and Liu 2008; Xu et al. 1999).

3 Detoxification of Noxious Foodstuffs by Histatins

The propensity of histatins to bind to negatively charged compounds is not limited to microbial membranes. In the oral cavity, many negatively charged molecules occur to which histatins can bind. This may have physiological relevant effects.

Binding of histatins to tannins, polyphenolic compounds from plant origin, has been implicated in detoxification of dietary tannins found in tea, wine, berries, and nuts. Binding of histatin 5 to tannic acid also blocked its inhibition of α-amylase suggesting a protective role of histatins in the enzymatic activity within the digestive tract (Bennick 2002).

4 Inhibition of Dental Plaque Formation by Histatins

Binding of histatins to hydroxyapatite, the principal mineral in dental enamel, may play a role in remineralisation of the teeth after acidic attack (Richardson et al. 1993). Binding to hydroxyapatite also plays a role in the formation of the pellicle, a protective layer of (glyco)proteins glycoproteins that covers the dental surfaces and inhibits the adherence of bacteria. Although the matter whether the histatins retain their antimicrobial activity after binding to hydroxyapatite has been debated (Xu et al. 1993; Yin et al. 2003), results from in vivo studies suggest that hydroxyapatite binding may play a role in the inhibition of plaque formation, either by direct killing or by reduced adherence (Paquette et al. 2002; Van Dyke et al. 2002).

Histatins also retain their candidacidal activity after adsorption onto poly(methyl methacrylate). Modifications of this polymer to improve its histatin 5-adsorbing properties with the long-term goal to produce PMMA-based dentures that are less susceptible to colonisation by *C. albicans* showed promising results in vitro (Edgerton et al. 1995; Yoshinari et al. 2006).

The strong binding of histatins to *Porphyromonas gingivalis*, a Gram-negative bacterium associated with periodontal disease, inhibits hemagglutination of this microorganism, supposedly by blocking adhesins important for adherence to oral surfaces (Murakami et al. 1992). Histatins also significantly reduced co-aggregation of *P. gingivalis* with *Streptococcus mitis*, a cariogenic member of dental plaque (Murakami et al. 1991), thus reducing its adherence to oral surfaces indirectly.

5 Anti-inflammatory Activity of Histatins

The ability of histatins to inhibit the activity of proteolytic enzymes from both microbial and host origins has been implicated in modulation of the immune response to oral infections (Basak et al. 1997; Gusman et al. 2001; Nishikata et al. 1991). However, there has been cast some doubt on whether histatins indeed are *bona fide* protease inhibitors. It has been demonstrated that the inhibitory activity of histatins gradually decreases during incubation with proteinases, because of proteolytic breakdown. In other words, histatins function as a substrate competing for the enzyme with the artificial substrates used for measuring protease activity (O'Brien-Simpson et al. 1998). Furthermore, inhibition of metalloproteinases by histatins has been attributed to their metal-binding properties. It is generally known that histidine-rich peptides have strong affinity for metal ions, because the imidazole group in the side chain of histidine is a chelator of metal ions. It is, therefore, not unexpected that histatins act as inhibitors of metalloproteinases, which require metal ions as cofactor for their enzymatic activity.

Alternatively, histatins have been implicated in immunomodulation by means of their LPS-binding properties, leading to suppression of the inflammatory induction of IL-6 and IL-8 from human gingival fibroblasts in vitro (Imatani et al. 2000; Sugiyama 1993).

6 Enzymatic Activity of Histatins

Serine proteases, esterases, and haloperoxidases each harbour a serine residue in their active site, of which the side-chain hydroxyl group acts as the nucleophile that attacks the substrate. This hydroxyl group is activated via proton abstraction by the imidazole side chain of an adjacent histidine residue, and the resulting imidazolium cation on its turn is stabilised by electrostatic interaction with the negatively charged side chain of a neighbouring aspartate residue (Hofmann et al. 2002). Although histatins do not possess the highly organised structures and specific binding pockets of proper enzymes, they do possess the necessary aspartate, histidine, and serine residues and the structural flexibility to position these in the proper conformation for enzymatic activity, at least in theory. Coordination of metal ions with the histidine side chains may stabilise such conformations and enhance the enzymatic activity considerably.

Enzymatic activity of histatins is poorly documented. We have observed that the use of the non-specific esterase substrate carboxy-DCFDA as membrane-integrity probe resulted in a high background signal due to the esterase activity of histatin 5 and that prolonged storage of highly purified histatin 5 in solution leads to (auto-catalytic?) degradation (unpublished results). Histatins do bind copper and zinc ions very well (Brewer and Lajoie 2000). After binding of copper ions, histatins display nuclease activity (Melino et al. 2006), which has been considered to contribute to their candidacidal activity.

Interestingly, in vitro studies showed that, under physiological concentrations of Cu^{2+}-ions, histatin 5 or histatin 8, and appropriate reductors such as ascorbate or cysteine, a copper-histatin complex was formed that produced hydrogen peroxide with high efficiency (Houghton and Nicholas 2009). By itself, hydrogen peroxide is not very toxic to microorganisms in the mouth, but, in theory, this reaction may well be suited to fuel the lactoperoxidase system, a well-defined antimicrobial system in saliva that uses hydrogen peroxide to enzymatically convert the abundant salivary thiocyanate into the bactericidal hypothiocyanite.

7 The Role of Histatins in Wound Healing

We found that histatins also play a prominent role in wound-healing processes in the mouth (Oudhoff et al. 2008). They achieve this by inducing the stretching and migration of epithelial cells at the edges of the wound, which results in a rapid re-epithelisation towards the centre of the wound. Although histatins are only secreted in saliva, they affect epithelial cells from skin tissue as well; in other words, licking your wounds may accelerate wound closure (Oudhoff et al. 2009).

Many antimicrobial peptides have a high therapeutic index, i.e. a high selectivity to microbial membranes over mammalian (host) membranes. We found that histatin 5, the most potent peptide from the histatin family, has no detectible lytic activity towards human cell membranes (Helmerhorst et al. 1999). This suggests that the interaction of histatins with human cells is governed by other molecular features than their interaction with microbial cells. The first observation confirming this hypothesis is that histatin 5, despite being the most potent antimicrobial histatin, showed little if any activity in the in vivo and ex vivo wound-healing models. On the other hand, histatins 1 and 2, with the lowest antimicrobial activity within the family, were among the most potent wound-healing stimulating histatins. The antimicrobial activity of histatin 5 has been ascribed to a non-specific electrostatic interaction with the microbial membrane caused by a positive charge and the ability to adopt an amphipathic structure. In contrast, in wound closure studies, all-D histatin 2 was completely inactive, and truncation analysis revealed a minimal active domain for stimulation of epithelial cells: SHREFPFYGDYGS (Figs. 1 and 3), residues 20–32 in histatin-1 numbering (Oudhoff et al. 2008, 2009). These two features are suggestive for the involvement of a specific histatin receptor on epithelial cells. There are more observations that point in this direction. Receptor-mediated activation of cell migration is generally accompanied by an active internalisation of the receptor–ligand complex. Indeed, the uptake of fluorescein-labelled histatin 1 by epithelial cells was almost completely abolished by depletion of the energy charge of the cells by lowering the incubation temperature to 4 °C or by pre-incubation with the energy poison sodium azide.

Pretreatment of epithelial cells with trypsin to strip them of membrane-bound surface-exposed proteins rendered them completely insensitive to histatin 1 and prevented the intracellular uptake of the fluorescein-labelled peptide (Oudhoff et al. 2008).

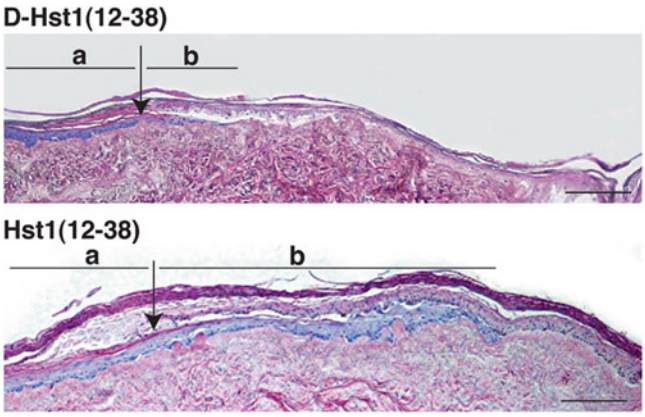

Fig. 3 *Histatin 2-stimulated re-epithelisation of a wound in a human skin model*. Representative micrographs of histatin 2-treated freeze-burn wounds in a validated human skin model, six days after wounding. The *arrows* indicate the boundaries of the wounds: (a) indicates intact epidermis, (b) indicates newly ingrown epidermis, and *bars* represent 250 μm. Epithelial cells are stained blue with hematoxylin and eosin. Histatin 2 (here represented as Hst1(12–38)) enhanced re-epithelisation of the artificial wound considerably (*lower panel*), in contrast to the all-D enantiomer of histatin 2 (here represented as D-Hst1(12–38)) that showed no enhancement of re-epithelisation over spontaneous ingrowth (*upper panel*). The stereospecificity of histatin 2-induced enhancement of re-epithelisation is indicative for the involvement of a receptor-mediated process

Activation of epithelial cells was not only energy and sequence dependent but also conformation dependent. Introduction of a conformational restraint into histatin 1 by head-to-tail cyclisation lead to a 1,000-fold increase in epithelial cell activation in vitro and ex vivo (Oudhoff et al. 2009).

Before we discuss the nature of this histatin receptor in more detail, we must realise that the prominent role of histatins in the wound-healing properties of saliva is unique in the animal kingdom. Histatins are only found in the saliva of humans and higher primates, so the wound-healing properties of saliva from other animals, well established for many years, must be attributed to different effector molecules. A major breakthrough in understanding the wound-healing properties of rodent saliva was reached with the identification of epidermal growth factor (EGF), which plays a crucial role in wound-healing processes such as cell proliferation, cell differentiation, and cell migration (Cohen 2008). EGF is also found in human saliva, and human epithelial cells do possess an EGF receptor (EGFR) at their surface. However, the concentration of EGF in human saliva is ~100,000 times lower than in rodent saliva, too low to play a significant role in human oral wound healing (Oudhoff et al. 2008). Nevertheless, human buccal epithelial cells could be activated by addition of recombinant human EGF, demonstrating that the EGF-driven machinery for activation of epithelial cells and stimulation of wound healing is present in the human mouth, but apparently, histatins have partially taken over this physiological role of EGF in human saliva.

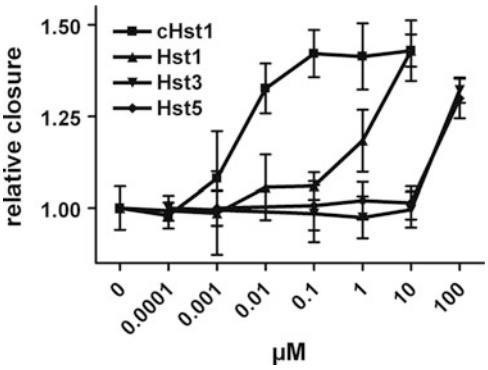

Fig. 4 *Effects of histatins on wound closure in vitro*. The three most abundant histatins in human parotid saliva were tested in scratch assays using confluent layers of human buccal epithelial cells grown on microscope slides as in vitro model for wound closure. Histatin 1 (*filled triangle*) was the most active, with 50 % of the maximal stimulatory effect around a concentration of 1 μM, well within the physiological concentration range in parotid saliva (around 2½ μM). Histatin 3 (*filled inverted triangle*) and histatin 5 (*filled diamond*) were completely inactive up to concentrations of 10 μM, way above their physiological concentrations (around 1 and 4 μM, respectively). Cyclisation of histatin 1 (*filled square*) resulted in a 1,000-fold increase in biological activity, with 50 % of the maximal stimulatory effect around a concentration of 1 nM. Enhancement of activity by cyclisation, i.e. the introduction of a conformational constraint within the peptide structure, is often regarded as an indication for the involvement of a ligand-receptor binding process

Histatins do not use the EGF receptor. Neither the specific EGFR inhibitor AG1478 nor SB203580, a specific inhibitor of the EGFR-linked p38MAPK signalling pathway, had any effect on the activity of histatin 2. On the other hand, pertussis toxin, a specific inhibitor of $G\alpha_1$-linked G-protein-coupled receptors, and U0126, a specific inhibitor of the GPCR-linked ERK1/2 signalling pathway, both completely inhibited the activity of histatin 2, without having any effect on the activity of rhEGF. In this respect, histatins are also unique; other human salivary antimicrobial peptides with wound-healing properties, β-defensins and the cathelicidin peptide LL-37 (even all-D LL-37), act through transactivation of the EGFR. Like EGF, these peptides induce cell proliferation besides cell migration.

Although histatins 3 and 5 activate epithelial cells to a much lesser extent (Fig. 4), they use the same receptor as histatins 1 and 2 for intracellular uptake. Uptake of histatin 5 was inhibited by histatin 2 and vice versa demonstrating that their binding affinities were comparable. Nevertheless, no antagonism between histatin 2 and histatin 5 was observed with respect to cell stimulating activity. This behaviour is not uncharacteristic for G-protein-coupled receptors. For several G-protein-coupled receptors an induced-fit model has been proposed, in which the ligand initially binds loosely to the receptor in its inactive form and subsequently induces the change of both ligand and receptor into the active form, leading to the formation of the ligand-receptor complex. This model explains the apparent discrepancy between the activity and the structural properties of cyclic histatin 1; initially, similar to its linear counterpart, this binds loosely and non-selectively to the inactive conformation of the

receptor. In the binding site then apparently conditions are present which favour the transition to the bioactive conformation of the peptide and, in concert, a transition of the receptor to its active conformation takes place. Peptides such as histatin 5, which are virtually inactive but yet internalised by the cell, can only perform the first step (loose binding to the inactive receptor), but cannot adopt the right conformation required to trigger a conformational change in the receptor to its active form. This initial binding is nevertheless sufficient for endocytosis, ostensibly similar to antagonist activities (Simmons et al. 1997).

8 Histatins: Two Functionally Different Subfamilies

The existence of two separate histatin genes has been enigmatic for many years. With regard to antimicrobial activity, the *HTN1* gene is virtually redundant; the contributions of histatins 1 and 2 to the total antimicrobial activity of the histatins present in human saliva are negligible. From an evolutionary viewpoint, the fact that the *HTN1* gene is nevertheless fully functional therefore must point to another function. Twenty-five years after the discovery of the histatins, this enigma was solved by our discovery that the *HTN1*-encoded histatins 1 and 2 possess high epithelial cell-migration-inducing activity (Oudhoff et al. 2009).

How specific are the histatin genes on a functional level? The antimicrobial activity of the histatin family is governed by the *HTN2*-encoded histatins 3–12, all expressing considerable antimicrobial activity. On molar basis, the *HTN1*-encoded histatins 1 and 2 make up around one third of the total histatin pool present in saliva, but, due to their low potency, their relative contribution to the total histatin antimicrobial activity adds up to less than 10 % (Oppenheim et al. 1986, 1988; Oudhoff et al. 2008). At high concentrations, the *HTN2*-encoded histatins 3 and 5 also show a considerable epithelial cell-migration-inducing activity in vitro; at a 30 μM concentration, histatin 3 reaches the same maximal stimulation as histatins 1 and 2 (Oudhoff et al. 2009). Up to 10 μM concentrations, histatins 3 and 5 were inactive (Oudhoff et al. manuscript in preparation). The physiological concentrations of histatins 3 and 5 in human parotid saliva are well below this threshold (1–4 μM). The *HTN1*-encoded histatins 1 and 2 are active at much lower concentrations: histatin 1 reaches maximal stimulation between 1 μM (50 %) and 10 μM (Oudhoff et al. manuscript in preparation). The physiological concentration of histatin 1 in human parotid saliva lies within this range (~2½ μM). Thus, the wound-healing activity of human parotid saliva can be attributed exclusively to the *HTN1*-encoded histatins, whereas the antimicrobial activity can be attributed almost completely to the *HTN2*-encoded histatins.

Several other properties of histatins can be correlated to either of the encoding genes. Binding of histatins to hydroxyapatite is mainly limited to histatin 1, due to its negatively charged C-terminus containing a phosphoserine residue (Richardson et al. 1993). Histatin 5 is a strong LPS binder and presumably has immunomodulatory properties, whereas histatin 2 shows little if any LPS binding and no immunomodulatory activity (Imatani et al. 2000; Oudhoff et al. 2009).

Obviously, it is beneficial to spread different properties implicated in innate immunity over different peptides, especially as these properties interfere with each other or even are counteractive. In theory, it allows a better fine-tuning of the innate immune response. The activity of a peptide with all these properties combined such as LL-37 is confined to very narrow concentration margins (Oudhoff et al. 2010). For instance, the ability of histatin 2 to induce epithelial cell migration at extreme high concentrations (100 μM) can only be beneficial when accompanied by its loss of cytolytic activity and immunomodulating properties. These are retained in histatin 5, which, in addition, may also assist histatin 2 in the wound-healing process by reducing fibrosis (and subsequent scarification) by inhibition of host collagenases.

Histatin-induced wound healing is a very young area of research, and there are not yet any data supporting the theory that the separation of wound healing and antimicrobial properties within the histatin family leads to cooperation between members of the two functionally different subfamilies. Although the concept is tempting, the slight overlaps in antimicrobial and cell-migration-inducing activities between the subfamilies appear to contradict this theory.

9 Histatins: Multifunctional Salivary Antimicrobial Peptides?

It is amazing how many different functional properties histatins manage to comprise within such small stretches of amino acids. According to many experiments in vitro, histatins may play a pivotal role in innate oral immunity. Their protective activity may cover many different areas, stretching from detoxification of noxious compounds to killing of invading microbes and from acceleration of wound healing to modulation of the immune response. The physiological importance of these various functional properties for an actual role in the innate immunity, however, for the most part still remains unclear. The working environment of the histatins, human saliva, is a very complex fluid containing literally hundreds of chemical compounds: ions, salivary (glyco)proteins and whatever compounds we may fancy to ingest, and hundreds of microbial species. Combined with the multifunctionality of histatins, this leads to a cornucopia of interactions that may interfere with the proposed roles in innate immunity. In principle, these could be beneficial (synergism); however, in most cases, these interactions only reduce the activity of histatins. The most obvious example is the abundance of host and microbial proteases in saliva that lead to a rapid breakdown of the histatins. This makes it extremely difficult to translate the activity of histatins in vitro to the situation in vivo. Several in vivo studies in animal models and patient studies have shown the therapeutic potential of histatins as antimicrobial agents (Paquette et al. 2002; Santarpia et al. 1991; Van Dyke et al. 2002; Welling et al. 2007). Yet, studies in vitro using physiological ionic-strength conditions show almost complete inactivation of histatins due to their high salt sensitivity (Edgerton et al. 1998; Helmerhorst et al. 1997; Xu et al. 1999). Does this mean that the membranolytic

activity of histatins observed in candidacidal assays in vitro plays a minor role in their physiological killing of *C. albicans* and that other antimicrobial properties emerge in vivo? The propensity of histatins, together with copper ions and suitable electron acceptors present in saliva, to generate hydrogen peroxide, which, in its turn, may fuel the antimicrobial lactoperoxidase system, could render such a scenario feasible.

Although histatins have been considered for many years as exceptional antimicrobial peptides, apart from their delayed action on the *C. albicans* membrane, at least in vitro, they behave very much like other membranolytic antimicrobial peptides. Nevertheless, histatins do possess a number of features that justify a special position within the group of antimicrobial peptides. Besides the ability to disturb microbial membranes, histatins display a large number of activities in vitro that have been implicated to innate immunity in humans. However, it is extremely difficult to evaluate whether a more or less artificial activity in vitro corresponds to a physiological function. Virtually, all antimicrobial activities discussed above lack solid evidence of physiologic relevance. Understanding the true role of histatins in the innate immunity of humans remains an important challenge for future research. Another striking difference with other peptides involved in innate immunity is that the interaction of histatins with microorganisms and with host cells is separated over two different subfamilies, each with its own coding gene. Altogether, this makes the histatins a unique group of multifunctional host defence peptides.

References

Basak A, Ernst B, Brewer D, Seidah NG, Munzer JS, Lazure C, Lajoie GA (1997) Histidine-rich human salivary peptides are inhibitors of proprotein convertases furin and PC7 but act as substrates for PC1. J Pept Res 49:596–603

Bennick A (2002) Interaction of plant polyphenols with salivary proteins. Crit Rev Oral Biol Med 13:184–196

Brant EC, Santarpia RP III, Pollock JJ (1990) The role of pH in salivary histidine-rich polypeptide anti-fungal germ tube inhibitory activity. Oral Microbiol Immunol 5:336–339

Brewer D, Lajoie G (2000) Evaluation of the metal binding properties of the histidine-rich antimicrobial peptides histatin 3 and 5 by electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 14:1736–1745

Brewer D, Lajoie G (2002) Structure-based design of potent histatin analogues. Biochemistry 41:5526–5536

Caldéron-Santiago M, Luque de Castro MD (2009) The dual trend in histatins research. Trends Anal Chem 28:1011–1018

Cohen S (2008) Origins of growth factors: NGF and EGF. J Biol Chem 283:33793–33797

Den Hertog AL, Wong Fong Sang HW, Kraayenhof R, Bolscher JGM, Van 't Hof W, Veerman ECI, Nieuw Amerongen AV (2004) Interactions of histatin 5 and histatin 5-derived peptides with liposome membranes: surface effects, translocation and permeabilization. Biochem J 379:665–672

Den Hertog AL, Van Marle J, Van Veen HA, Van 't Hof W, Bolscher JGM, Veerman ECI, Nieuw Amerongen AV (2005) Candidacidal effect of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. Biochem J 388:689–695

Edgerton M, Raj PA, Levine MJ (1995) Surface-modified poly(methyl methacrylate) enhances adsorption and retains anticandidal activities of salivary histatin 5. J Biomed Mater Res 29:1277–1286

Edgerton M, Koshlukova SE, Lo TE, Chrzan BG, Straubinger RM, Raj PA (1998) Candidacidal activity of salivary histatins identification of a histatin 5-binding protein on *Candida albicans*. J Biol Chem 273:20438–20447

Edgerton M, Koshlukova SE, Araujo MW, Patel RC, Dong J, Bruenn JA (2000) Salivary histatin 5 and human neutrophil defensin 1 kill *Candida albicans* via shared pathways. Antimicrob Agents Chemother 44:3310–3316

Gusman H, Travis J, Helmerhorst EJ, Potempa J, Troxler RF, Oppenheim FG (2001) Salivary histatin5 is an inhibitor of both host and bacterial enzymes implicated in periodontal disease. Infect Immun 69:1402–1408

Helmerhorst EJ, Van 't Hof W, Veerman ECI, Simoons-Smit I, Nieuw Amerongen AV (1997) Synthetic histatin analogs with broad spectrum antimicrobial activity. Biochem J 326:39–45

Helmerhorst EJ, Reijnders IM, Van 't Hof W, Veerman ECI, Nieuw Amerongen AV (1999) A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. FEBS Lett 449:105–110

Helmerhorst EJ, Van 't Hof W, Breeuwer P, Veerman ECI, Abee T, Troxler RF, Nieuw Amerongen AV, Oppenheim FG (2001) Characterization of histatin 5 with respect to amphipathicity, hydrophobicity and effects on cell and mitochondrial membrane integrity excludes a candidacidal mechanism of pore formation. J Biol Chem 276:5643–5649

Hofmann A, Grella M, Botos I, Filipowicz W, Wlodawer A (2002) Crystal structures of the semireduced and inhibitor-bound forms of cyclic nucleotide phosphodiesterase from *Arabidopsis thaliana*. J Biol Chem 277:1419–1425

Houghton EA, Nicholas KM (2009) *In vitro* reactive oxygen species production by histatins and copper (I, II). J Biol Inorg Chem 14:243–251

Imatani T, Kato T, Minaguchi K, Okuda K (2000) Histatin 5 inhibits inflammatory cytokine induction from human gingival fibroblasts by *Porphyromonas gingivalis*. Oral Microbiol Immunol 15:378–382

Isola R, Isola M, Diaz G, Conti G, Lantini MS, Riva A (2007) Histatin-induced alterations in *Candida albicans*: a microscopic and submicroscopic comparison. Microsc Res Tech 70:607–616

Jang WS, Bajwa JS, Sun JN, Edgerton M (2010) Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. Mol Microbiol 77:54–370

Kavanagh K, Dowd S (2004) Histatins; antimicrobial peptides with therapeutic potential. J Pharm Pharmacol 56:285–289

Koshlukova SE, Lloyd TL, Araujo MW, Edgerton M (1999) Salivary histatin 5 induces non-lytic release of ATP from *Candida albicans* leading to cell death. J Biol Chem 274:18872–18879

Luque-Ortega JR, Van 't Hof W, Veerman ECI, Saugar JM, Rivas L (2008) Human antimicrobial peptide histatin5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in *Leishmania*. FASEB J 22:1817–1828

Mackay BJ, Denepitiya L, Jacono VJ, Krost SB, Pollock JJ (1984a) Growth-inhibitory and bactericidal effects of human parotid salivary histidine-rich polypeptides on *Streptococcus mutans*. Infect Immun 44:695–701

Mackay BJ, Pollock JJ, Jacono VJ, Baum BJ (1984b) Isolation of milligram quantities of a group histidine-rich polypeptides from human parotid saliva. Infect Immun 44:688–694

Melino S, Gallo M, Trotta E, Mondello F, Paci M, Petruzzelli R (2006) Metal-binding and nuclease activity of an antimicrobial peptide analogue of the salivary histatin 5. Biochemistry 45:15373–15383

Mochon AB, Liu H (2008) The antimicrobial peptide histatin 5 causes a spatially restricted disruption on the *Candida albicans* surface, allowing rapid entry of the peptide into the cytoplasm. PLOS Pathog 4(10):e1000190. doi:101371/journalppat1000190

Murakami Y, Nagat H, Amano A, Takagaki M, Shizukuishi S, Tsunemitsu A, Aimoto S (1991) Inhibitory effects of human salivary histatins and lysozyme on coaggregation between *Porphyromonas gingivalis* and *Streptococcus mitis*. Infect Immun 59:3284–3286

Murakami Y, Tamagawa H, Shizukuishi S, Tsunemitsu A, Aimoto S (1992) Biological role of an arginine residue present in a histidine-rich peptide which inhibits hemagglutination of *Porphyromonas gingivalis*. FEMS Microbiol Lett 98:201–204

Nishikata M, Kanehira T, Oh H, Tani H, Tazaki M, Kuboki Y (1991) Salivary histatin as an inhibitor of a protease produced by the oral bacterium *Bacteroides gingivalis*. Biochem Biophys Res Commun 174:625–630

O’Brien-Simpson NM, Dashper SG, Reynolds EC (1998) Histatin 5 is a substrate and not an inhibitor of the Arg- and Lys-specific proteinases of *Porphyromonas gingivalis*. Biochem Biophys Res Commun 250:474–478

Oppenheim FG, Yang Y-C, Diamond RD, Hyslop D, Offner GD, Troxler RF (1986) The primary structure and functional characterization of the neutral histidine-rich polypeptide from human parotid secretion. J Biol Chem 261:1177–1182

Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. J Biol Chem 263:7472–7477

Oudhoff MJ, Bolscher JGM, Nazmi K, Kalay H, Van ‘t Hof W, Nieuw Amerongen AV, Veerman ECI (2008) Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. FASEB J 22:3805–3812

Oudhoff MJ, Kroeze K, Nazmi K, Van den Keijbus P, Van ‘t Hof W, Fernandez-Borja M, Hordijk PL, Gibbs S, Bolscher JGM, Veerman ECI (2009) Structure-activity analysis of histatin, a potent wound healing peptide from human saliva: cyclization of histatin potentiates molar activity 1,000-fold. FASEB J 23:3928–3935

Oudhoff MJ, Blaauboer ME, Nazmi K, Scheres N, Bolscher JGM, Veerman ECI (2010) The role of salivary histatin and the human cathelicidin LL-37 in wound healing and innate immunity. Biol Chem 391:541–548

Paquette DW, Simpson DM, Friden P, Braman V, Williams RC (2002) Safety and clinical effects of topical histatin gels in humans with experimental gingivitis. J Clin Periodontol 29:1051–1058

Pollock JJ, Denepitiya L, Mackay BJ, Jacono VJ (1984) Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptides on *Candida albicans*. Infect Immun 44:702–707

Raj PA, Edgerton M, Levine MJ (1990) Salivary histatin 5: dependence of sequence, chain length and helical conformation for candidacidal activity. J Biol Chem 265:3898–3905

Raj PA, Soni S-D, Levine MJ (1994) Membrane-induced helical conformation of an active candidacidal fragment of salivary histatins. J Biol Chem 269:9610–9616

Raj PA, Marcus E, Sukumaran DK (1998) Structure of human salivary histatin 5 in aqueous and nonaqueous solutions. Biopolymers 45:51–67

Richardson CF, Johnsson M, Raj PA, Levine MJ, Nancollas GH (1993) The influence of histatin 5 fragments on the mineralization of hydroxyapatite. Arch Oral Biol 38:997–1002

Rothstein DM, Spacciapoli P, Tran LT, Xu T, Roberts FD, Dalla Serra M, Buxton DK, Oppenheim FG, Friden P (2001) Anticandida activity is retained in P-113, a 12-amino-acid fragment of histatin 5. Antimicrob Agents Chemother 45:1367–1373

Ruissen ALA, Groenink J, Helmerhorst EJ, Walgreen-Weterings E, Van ‘t Hof W, Veerman ECI, Nieuw Amerongen AV (2001) Effects of histatin 5 and derived peptides on *Candida albicans*. Biochem J 356:361–368

Ruissen ALA, Groenink J, Krijtenberg P, Walgreen-Weterings E, Van ‘t Hof W, Veerman ECI, Nieuw Amerongen AV (2003) Internalisation and degradation of histatin 5 by *Candida albicans*. Biol Chem 384:183–190

Sabatini LM, Azen EA (1989) Histatins, a family of salivary histidine-rich proteins, are encoded by at least two *loci* HIS1 and HIS2. Biochem Biophys Res Commun 160:495–502

Sabatini LM, Warner TF, Saitoh E, Azen EA (1989) Tissue distribution of RNAs for cystatins, histatins, statherin and proline-rich salivary proteins in humans and macaques. J Dent Res 68:1138–1145

Santarpia RP III, Pollock JJ, Renner RP, Gwinnett AJ (1991) *In vivo* antifungal efficacy of salivary histidine-rich polypeptides: preliminary findings in a denture stomatitis model system. J Prosthet Dent 66:693–699

Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, Buser R, Wells TN, Proudfoot AE (1997) Potent inhibition of HIV-infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science 276:276–279

Sugiyama K (1993) Anti-lipopolysaccharide activity of histatins, peptides from human saliva. Experientia 49:1095–1097

Tsai H, Raj PA, Bobek LA (1996) Candidacidal activity of recombinant human salivary histatin 5 and variants. Infect Immun 64:5000–5007

Van 't Hof W, Veerman ECI, Helmerhorst EJ, Nieuw Amerongen AV (2001) Antimicrobial peptides, properties and applicability. Biol Chem 382:597–619

Van Dyke T, Paquette D, Grossi S, Braman V, Massaro J, D'Agostino R, Dibart S, Friden P (2002) Clinical and microbial evaluation of a histatin-containing mouthrinse in humans with experimental gingivitis: a phase-2 multi-center study. J Clin Periodontol 29:168–176

Vanderspek JC, Offner GD, Troxler RF, Oppenheim FG (1990) Molecular cloning of human submandibular histatins. Arch Oral Biol 35:137–143

Veerman ECI, Nazmi K, Van 't Hof W, Bolscher JGM, Den Hertog AL, Nieuw Amerongen AV (2004) Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5. Biochem J 381:447–452

Veerman ECI, Valentijn-Benz M, Nazmi K, Ruissen ALA, Walgreen-Weterings E, Van Marle J, Doust AB, Van 't Hof W, Bolscher JGM, Nieuw Amerongen AV (2007) Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane. J Biol Chem 282:18831–18841

Welling MM, Brouwer CP, Van 't Hof W, Veerman ECI, Nieuw Amerongen AV (2007) Histatin-derived monomeric and dimeric synthetic peptides show strong bactericidal activity towards multidrug-resistant *Staphylococcus aureus in vivo*. Antimicrob Agents Chemother 51:3416–3419

Xu T, Telser E, Troxler RF, Oppenheim FG (1990) Primary structure and anticandidal activity of the major histatin from parotid secretion of the subhuman primate *Macaca fascicularis*. J Dent Res 69:1717–1723

Xu T, Choi YJ, Saxer C, Oppenheim FG (1993) Hydroxyapatite adsorption and candidacidal activity of histatins. J Dental Res 72:322 (Abstract)

Xu Y, Ambudkar I, Yamagishi H, Swaim W, Walsh TJ, O'Connell BC (1999) Histatin 3-mediated killing of *Candida albicans*: effect of extracellular salt concentration on binding and internalization. Antimicrob Agents Chemother 43:2256–2262

Yin A, Margolis HC, Grogan J, Yao Y, Troxler RF, Oppenheim FG (2003) Physical parameters of hydroxyapatite adsorption and effect on candidacidal activity of histatins. Arch Oral Biol 48:361–368

Yoshinari M, Kato T, Matsuzaka K, Hayakawa T, Inoue T, Oda Y, Okuda K, Shimono M (2006) Adsorption behavior of antimicrobial peptide histatin 5 on PMMA. J Biomed Mater Res B Appl Biomater 77:47–54