Anticandidal Activity of Major Human Salivary Histatins

TAO XU,1 STUART M. LEVITZ,2 RICHARD D. DIAMOND,2 AND FRANK G. OPPENHEIM1,3*

Department of Periodontology and Oral Biology, Goldman School of Graduate Dentistry,1 Section of Infectious Diseases, Evans Memorial Department of Clinical Research and Department of Medicine,2 and Department of Biochemistry,3 School of Medicine, Boston University Medical Center, Boston, Massachusetts 02118

Received 1 March 1991/Accepted 10 May 1991

We have previously shown that histatins 1, 3, and 5 are homologous, histidine-rich proteins present in human parotid and submandibular secretions which contain 38, 32, and 24 amino acids, respectively. Interest in these proteins stems from the fact that histatins exhibit candidacidal and candidastatic activities. The goal of the present investigation was a detailed functional characterization of these anticandidal activities of histatins at the levels of killing of blastoconidia, killing of germinated cells, and inhibition of germination by using three bioassays. Candidacidal activities were evaluated at several ionic strengths, in the presence of different monovalent and divalent ions, and at multiple pH values. In addition, the susceptibility of Candida albicans in different growth phases to histatins was investigated. While all three major human histatins demonstrated candidacidal activities, they differed in their abilities to kill blastoconidia and germinated cells, with histatin 5 being the most active, histatin 3 showing moderate activity, and histatin 1 exhibiting the lowest level of activity. For the inhibition of germination, however, histatin 3 exhibited more activity than either histatin 1 or histatin 5. The candidacidal activity of histatins was inversely proportional to both the ionic strength and the divalent cation concentration in the medium. Stepwise reduction of the pH of the assay medium enhanced the candidacidal activities of histatins 1 and 3, while the activity of histatin 5 was pH independent over the range of pHs 4 to 8. C. albicans in log-phase growth was more susceptible to histatins 1 and 3 than cells in stationary phase. Cells in either growth phase were still more vulnerable to histatin 5 than to histatins 1 and 3. The results obtained establish the functional relationship of the major histatins with respect to both their fungicidal and fungistatic activities and provide insights into their activities under ionic and pH conditions likely to be encountered in vivo in the oral cavity. Moreover, the data point towards possible mechanisms responsible for the anticandidal activities of histatins.

Oral candidiasis is a relatively uncommon oral infection in the general population despite the fact that Candida albicans can be recovered from healthy individuals in over 50% of cases (12, 24). The host defense system preventing C. albicans from attachment to and colonization of the skin or mucosal surfaces is believed to comprise the immune system and nonimmune defense functions (3, 16). Recently, a group of antifungal polypeptides in the oral cavity (18–20) has been identified. This oral antifungal defense is based on the oral exocrine system and comprises a group of closely related histidine-rich salivary proteins named histatins. Human histatins represent a family of low-molecular-weight proteins which display a considerable degree of sequence homology (25). The predominant human histatins are histatin 1, histatin 3, and histatin 5, with molecular weights of 4,929, 4,063, and 3,037, respectively (Fig. 1).

We have previously characterized the major and minor components of the human histatin family and reported on their primary antifungal activity (18, 25). Since C. albicans is a dimorphic fungus, both its yeast (blastoconidial) and hyphal (germinated) forms and their conversion have to be considered in antifungal investigations. While the germinated form is considered to be more invasive, most of the C. albicans isolates harvested from the oral cavities of healthy individuals appear to be in the blastoconidial form (1, 6, 16). To evaluate the antifungal spectrum of histatins, we have therefore developed three bioassays which allow for the measurement of candidacidal activity against either blastoconidia or germinated cells and the candidastatic activity exerted by the inhibition of germination (26). The present work describes, in detail, the results obtained with these three assays with purified human histatin 1, histatin 3, and histatin 5. Considering the variability of the oral environment, a variety of modified buffer conditions were investigated with regard to their effects on the candidacidal activity of histatins. These conditions included variations in ionic strength, ionic composition, and pH. In addition, the susceptibility of C. albicans in different growth phases to histatins was investigated.

MATERIALS AND METHODS

Isolation of histatins. Human parotid secretion from healthy adults was stimulated with sour lemon candies, collected with Curby cups in ice-chilled graduated cylinders, pooled, dialyzed, and lyophilized as previously described (17). The total protein in a human parotid secretion sample was subjected to fractionation on a Bio-Gel P-2 column (Bio-Rad Laboratories, Richmond, Calif.) developed in 0.05 M ammonium formate buffer, pH 4.0. The protein fraction enriched with histatins was further purified by using reversed-phase high-performance liquid chromatography on a C18 column as previously reported (18). Purified histatins were evaporated to dryness, dissolved in deionized water, quantified by amino acid analysis, lyophilized, and stored at −20°C until used.

C. albicans. Two well-described C. albicans strains were used in three bioassays. Strain B311 was originally isolated from a patient afflicted with systemic candidiasis (7), while strain ATCC 44505 was originally isolated from the human oral cavity (15). Cultures were stored at 4°C on Sabouraud

* Corresponding author.
dextrose agar plates (Difco Laboratories, Detroit, Mich.) until used. Stationary-phase-growth cells were obtained following growth at 30°C for 18 h on Sabouraud dextrose agar plates. Colonies were harvested and suspended in 10 mM potassium phosphate buffer (PPB), pH 7.4.

To initiate log-phase growth, a sample of stock C. albicans was suspended in Sabouraud dextrose broth (Difco) and incubated at 30°C in a shaking water bath. The growth phase was determined by taking aliquots of the culture at 1-h intervals to monitor the optical density at 560 nm. Early log phase was obtained at 4 to 6 h and was indicated by an optical density of 0.6. Log-phase cells were harvested and utilized in the blastoconidium killing assay in a manner identical to that described for stationary-phase cells. A final concentration of 10^5 cells per ml (either stationary- or log-phase fungus) was used in all assays.

**Suspension buffers.** The standard suspension buffer utilized in the blastoconidium killing assay was 10 mM PPB, pH 7.4. To evaluate the influence of ionic strength on blastoconidium killing, 10-fold-diluted (1 mM) and 10-fold-concentrated (100 mM) PPB were used as alternate suspension buffers.

To evaluate the influence of several ion species on the antifungal effects of histatins, four salts, KCl, CaCl_2, MgCl_2, and MgSO_4, were chosen. Because of the potential interaction of calcium with phosphate ions, an alternate suspension buffer, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.4; Sigma Chemical Co., St. Louis, Mo.), was utilized. Salts were added at concentrations equal to the concentration of HEPES, e.g., 1 mM KCl in 1 mM HEPES. To evaluate the effectiveness of histatins over the possible pH range occurring in an oral cavity, PPB was prepared at pHs 4.0, 5.0, 6.0, 7.0, and 8.0. All chemicals used in experiments were obtained from Fisher Scientific (Pittsburgh, Pa.), unless otherwise specified.

**Bioassays.** Three assays were used to evaluate the effects of histatins on the killing of blastoconidia, on the killing of germinated cells, and on the inhibition of germination of C. albicans as previously described (26).

For the blastoconidium killing assay, 50-μl aliquots of cells (2 × 10^5 cells per ml) diluted in suspension buffer were allowed to attach to a polystyrene 96-well microtiter plate (Costar, Cambridge, Mass.) for 15 min at room temperature; they were then incubated with an equal volume of histatin 1, 3, or 5 in suspension buffer for 1 h at 37°C. Control experiments were carried out in the absence of histatins. After incubation, wells were washed three times by centrifugation at 1,000 × g for 5 min and covered with aliquots of molten Sabouraud dextrose broth (Difco) containing 2% agarose (Sigma) at 45°C, and the plate was incubated at 30°C for 8 h. Under such conditions, live cells will divide and begin to form colonies while dead cells will remain as single cells. To determine the percentage of blastoconidia killed, a total of 100 single cells and/or colonies was counted under a Nikon inverted microscope at 400× magnification and the extent of killing (expressed as a percentage) was calculated by the following formula: [1 − (number of colonies in treated sample/number of colonies in control)] × 100.

For the germinated-cell-killing assay, 50-μl aliquots of cells (10^5 cells per ml) were preincubated in RPMI 1640 ( GibCO-BRL Life Technologies, Inc., Gaithersburg, Md.) buffered with 10 mM HEPES, pH 7.4, in a 96-well microtiter plate at 37°C for 3 h. Such conditions routinely result in over 95% germination. After incubation, the germinated cells were washed three times by centrifugation, as described for the blastoconidium killing assay, and incubated with histatin 1, 3, or 5 in PPB at 37°C for 1 h. Wells were then washed three times by centrifugation, overlaid with molten Sabouraud dextrose broth containing 2% agarose at 45°C, and incubated at 25°C for 10 h. Under these conditions, colonies will form from live germinated cells while dead germinated cells show no morphological changes. The percentage of germinated cells killed was calculated as described for the blastoconidium killing assay.

For the inhibition-of-germination assay, 50-μl aliquots of blastoconidia in germination medium (RPMI 1640, pH 7.4; 2 × 10^5 cells per ml) were added to a 96-well microtiter plate and incubated with an equal volume of a test protein at 37°C for 3 h. A total of 100 cells (germinated cells and/or blastoconidia) were counted by using an inverted microscope at 400× magnification. In this assay, the inhibition (expressed as a percentage) was calculated by the following formula: [1 − (percent germination in treated sample/percent germination in control)] × 100. In the absence of test proteins (the control), germination was always 95% or greater. In addition, to determine whether those cells which did not germinate were dead or alive in this assay, the wells were washed by centrifugation to remove both histatin and RPMI 1640, overlaid with molten Sabouraud dextrose broth containing agarose, and incubated as described above. The ungerminated single cells formed colonies after incubation, verifying that this assay measures the inhibition of germination.

Complete dose-response curves were produced for strain B311 in three assays, namely, assays for the killing of blastoconidia, the killing of germinated cells, and the inhibition of germination. For these purposes, histatins were serially diluted, from a high concentration of 216 nmol/ml to a low concentration of 1.7 nmol/ml.

**Statistical analysis.** Data were obtained by calculating the means and standard deviations from triplicate assays. From the dose-response relationship, doses effecting a 50% killing (LD₅₀) or a 50% inhibition of germination (ID₅₀) were determined by probit transformation (5). The Newman-Keuls test was employed to determine whether data differed significantly among different histatins, protein compositions, and ionic strengths (27).
RESULTS

Killing of blastoconidia. The three major histatins killed blastoconidia of strain B311 effectively at concentrations equal to or greater than 6.8 nmol/ml (Fig. 2A). At concentrations less than 6.8 nmol/ml, differences among the histatins became evident. Histatin 5 was the most active component, while histatin 3 exhibited intermediate activity and histatin 1 showed the least activity. These differences were clearly reflected in the LD_{50}s (Table 1) of 2.2, 4.6, and 5.8 nmol/ml for histatins 5, 3, and 1, respectively. Interestingly, the corresponding LD_{50}s with oral-derived strain ATCC 44505 were 2.0, 4.2, and 6.3 nmol/ml (26), indicating negligible differences between the two Candida strains tested (Table 1).

Killing of germinated cells. The overall patterns of the dose-response curve for the killing of germinated cells were similar to that obtained for the killing of blastoconidia of strain B311. However, higher concentrations of histatins were required to kill germinated cells than were required for killing blastoconidia (Fig. 2B). While the order of effectiveness of the three histatins in killing germinated cells was identical to that described for killing blastoconidia, histatin 1 was dramatically less effective than histatins 3 and 5, as indicated by their LD_{50}s of 74.2, 13.5, and 8.5 nmol/ml, respectively (Table 1). The effects of histatins 1, 3, and 5 on killing germinated cells of strain ATCC 44505 (26) were similar to those obtained with strain B311 (Table 1).

Inhibition of germination. The dose-response curves for the inhibition of germination (Fig. 2C) were much steeper than the dose-response curves for both killing assays (Fig. 2A and B). Histatins at a concentration of 108 nmol/ml effected almost complete inhibition of germination, while at 13.5 nmol/ml less than 20% inhibition was measured. This candidastatic activity differed from the candidacidal patterns with respect to the rank orders of the three histatins. Histatin 3 was the most active candidastatic component, while histatin 5 exhibited the greatest candidacidal activity (Table 1). There were no significant differences in the ID_{50}s between strains B311 and ATCC 44505 (Table 1).

Effects of various suspension buffer compositions. The abilities of the three major histatins to kill blastoconidia were examined with various ionic strengths and ionic species. Under virtually all of the conditions tested, there were no significant differences among the three histatins (Fig. 3). Regardless of the ionic species present in the suspension buffer, increasing the concentration of ions from 10 to 100 mM resulted in a reduction of the killing abilities of all three histatins (Fig. 3B and C). When either PPB or HEPES buffer was used, identical activity levels were observed at all three molarities tested, allowing for the substitution of the latter buffer in the experiments utilizing divalent ions. The addition of the monovalent cation/anion KCl had no effect on the killing of blastoconidia. On the other hand, the addition of divalent salts, such as CaCl_{2}, MgCl_{2}, and MgSO_{4}, lowered the candidacidal activities of histatins. The statistical analysis with the Newman-Keuls test (27) showed that the differ-

**TABLE 1. Comparison of anticanidal activities of human histatins 1, 3, and 5**

<table>
<thead>
<tr>
<th>Assay and histatin</th>
<th>Activity against C. albicans strain*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B311</td>
</tr>
<tr>
<td>Blastoconidium killing</td>
<td></td>
</tr>
<tr>
<td>Histatin 1</td>
<td>5.8</td>
</tr>
<tr>
<td>Histatin 3</td>
<td>4.6</td>
</tr>
<tr>
<td>Histatin 5</td>
<td>2.2</td>
</tr>
<tr>
<td>Germinated-cell killing</td>
<td></td>
</tr>
<tr>
<td>Histatin 1</td>
<td>74.2</td>
</tr>
<tr>
<td>Histatin 3</td>
<td>13.5</td>
</tr>
<tr>
<td>Histatin 5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Values are given in nanomoles per milliliter of protein required for the LD_{50} or ID_{50}. Values in parentheses represent the 95% confidence interval of the LD_{50} or ID_{50}. No overlap of 95% confidence intervals indicates statistically significant differences.

* Data from reference 26.
ences in the ionic strengths stated above and in mono- and divalent ions were all significant at $P$ values of 0.05 or lower.

The ability of histatins to kill blastoconidia at multiple pH values was investigated, and the LD$_{50}$s for pHs 4.0 through 8.0 were determined (Fig. 4). While the activity of histatin 5 remained nearly constant over the pH range tested, the activities of histatins 1 and 3 were approximately 5- to 10-fold greater at pHs 4 and 5 than at pHs 7 and 8.

**Log-phase C. albicans.** Log-phase-growth C. albicans cells were more susceptible than stationary-phase cells with regard to killing by histatins 1 and 3 (Table 2). In contrast, histatin 5, the most active of the major histatins, killed cells of both growth phases with approximately equal efficiency.

![Graph showing % Killing vs pH for different ionic concentrations](image)

**DISCUSSION**

The data presented here demonstrate clear functional differences among human histatins 1, 3, and 5 with respect to both candidacidal and candidastatic activities, despite the fact that histatins exhibit a high degree of sequence homology (18, 25). In the candidacidal assays, histatin 5 was the most effective protein and histatin 1 was the least active protein. In contrast, histatin 3 was the most potent inhibitor of germination. The mechanisms responsible for the candidacidal and candidastatic activities appear to be different since the effectiveness of histatins differs so clearly in the respective assays. In addition, the differences in the potency of the various histatins within the same assay are most likely related to their structural differences, particularly those associated with their carboxyl-terminal domains.

This study explored the candidacidal effects of human histatins under a variety of conditions known to occur in the oral cavity, such as low ionic strength, the presence of divalent ions, and low pH. Interestingly, deviations from the standard assay conditions, such as raising ionic strengths and introducing divalent cations, resulted in diminished candidacidal activity. It is noteworthy that saliva is a hypotonic body fluid. Its ionic strength is on average 10 times that of serum, and divalent cations are present in saliva at 1 mM concentration or less (23). It is feasible that, as the ionic strength increases, the competition for cell surface sites increases, thereby lessening the interaction between histatins and blastoconidia, which may result in a decline in candidacidal activity.

**TABLE 2. Effects of human histatins on the killing of C. albicans in different growth phases**

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Histatin 1</th>
<th>Histatin 3</th>
<th>Histatin 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary</td>
<td>5.8 (5.5-6.1)</td>
<td>4.6 (4.3-4.8)</td>
<td>2.2 (1.7-2.5)</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>4.0 (3.7-4.4)</td>
<td>2.8 (2.6-3.0)</td>
<td>2.1 (1.7-2.3)</td>
</tr>
</tbody>
</table>

* Values given represent LD$_{50}$s. Values in parentheses represent 95% confidence intervals of the LD$_{50}$s. No overlap of 95% confidence intervals indicates statistically significant differences.
The prevailing pH in the oral cavity is dictated by that of saliva, which ranges between pHs 5 and 8 (23). On the tooth surface, however, pH values can be significantly lower because of the metabolic activities of cariogenic microorganisms (14). Our results indicate that low pH favorably influences the candidacidal effects. It is interesting that when C. albicans was incubated with histatins at pH values less than 7.0, the magnitude of the candidacidal activity increased. In particular, histatins 1 and 3 showed pH-dependent profiles. Plots of the LD₅₀ versus the pH revealed sigmoid curves such as those found in classical titration curves (Fig. 4). The observation that histatins 1 and 3 were more active at acidic pHs suggests the presence of available titratable groups in the carboxyl-terminal domain. The pH-dependent changes observed between pHs 5 and 7 for histatins 1 and 3 suggest a potential contribution from histidine residues present within histatins. The fact, however, that all three histatins contain seven histidine residues at identical positions makes it unlikely that the titration of histidine imidazole groups is solely responsible for the pH dependence observed. Furthermore, histatin 5 showed no dependence with respect to pH. It is more likely that acidic residues in the carboxyl-terminal domain account for the pH-dependent activity of histatins 1 and 3. This is supported by the fact that histatin 1, with three acidic residues in the carboxyl-terminal domain (Glu-23, Asp-29, and Asp-37), showed a greater pH dependence than histatin 3, which contains only one acidic residue (Asp-31). Histatin 5, on the other hand, showed virtually no pH dependence and does not possess the acidic residue-containing, carboxyl-terminal domains of histatins 1 and 3 (Fig. 1). Furthermore, the data obtained indicate that major histatins are candidacidal for fungal cells in the stationary phase as well as cells growing in the log phase (Table 2). Differences in affinities of binding of histatins to the cell surfaces could again relate to the acidic residues present in the carboxyl-terminal domains of histatins 1 and 3, which are absent in histatin 5. These protein moieties could interact differentially with the cell surfaces of stationary- and log-phase C. albicans.

While there is only limited information on the higher-order structure of the histatin family, one interesting observation on histatin 5 has been reported by Raj et al. (21). Their investigation showed that synthetic histatin 5 analogs which assumed an α-helix structure in hydrophobic solutions also exhibited candidacidal activity. It is possible that the environmental parameters, such as theionic strength, the ionic species, and the pH, affect the secondary structure of histatins, thereby influencing their function.

Another naturally occurring family of polypeptides with known antifungal activity are the defensins, a group of arginine- and cysteine-rich polypeptides isolated from mammalian phagocytes (4, 9). Like histatins, defensins have antifungal activities, have low molecular weights, and are cationic in nature. Therefore, one might expect that changes in the ionic strength and modifications of ionic composition, pH, and growth phase might affect blastocandidum susceptibility to defensins in a manner similar to that to histatins. This was indeed the case in terms of ionic strength, divalent cations (calcium and magnesium), and log-phase susceptibility to the candidacidal activity (10, 11, 22). Despite these similarities, the histatins differ from defensins structurally and functionally. Candidacidal effects for defensins can be observed at nanomolar concentrations (10), whereas micromolar concentrations are required for histatins. The most significant differences relate to their biosynthetic origin, amino acid composition, and magnitude of biologic activity.

Unlike defensins, which require prior degranulation and release from tissue-bound cells to interact with fungal cells, histatins' natural fate is their continuous secretion and release into human parotid and submandibular secretions. The distinct differences in their effectiveness in killing C. albicans appear to be related to differences in their sites of action. The high potency of defensins is clearly required within tissue where their release leads to a rapid dilution into interstitial fluid. Histatins, on the other hand, do not require a high potency since they represent a major secretory product of saliva, which is constantly replenished in the oral cavity. Furthermore, specific binding of histatins to the host and/or pathogen could lead to an increased concentration in specific local oral microenvironments.

The full physiological value of histatins is difficult to establish at this juncture. Despite the presence of histatins at a concentration range of between 1 and 30 nmol/ml in salivary glandular secretions (2), their concentration in whole saliva or oral fluid is not known. The proteolytic potential of whole saliva could lead to the rapid degradation of histatins after their release into the oral cavity. Since the proteolytic potential of whole saliva is highly variable, histatins' candidacidal activity could become limited under certain conditions. High oral proteolytic activity is clearly related to an increase in plaque accumulation and concomitant gingivitis, which are more likely to occur in compromised patients at high risk for oral candidiasis (8, 13). Further studies are required to determine whether transient reductions in the levels of histatins in the oral environment are responsible for the onset of oral candidiasis. Indirect evidence to support a role for histatins in defense against C. albicans infection is derived from clinical observations with denture wearers. Denture stomatitis-affected patients reveal candidal infection of mucosal surfaces which are shielded from exposure to fresh saliva. A unique feature of histatins is that they are not serum proteins, such as immunoglobulins, or tissue-bound proteins, such as defensins, but are proteins freshly occurring in an exocrine secretion. The constant presence of histatins in the oral environment allows for the direct interaction with fungal cells even before such cells can attach to the epithelial surfaces, which may result in the prevention of infection. In addition, the direct interaction of histatins with C. albicans may lead to cell death or inhibit their germination. In the spectrum of antifungal control in the oral cavity, histatins are uniquely suited to represent the first line of host defense against oral candidiasis.

ACKNOWLEDGMENTS

This research was supported in part by Public Health Service grants DE 05672 and DE 07652 from the National Institute of Dental Research and AI 25780 and AI 15338 from the National Institute of Allergy and Infectious Diseases.

The constructive discussion of the manuscript by Mark S. Lamkin is greatly appreciated.

REFERENCES