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Salivary secretory leukocyte protease inhibitor increases in HIV infection*

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BACKGROUND: Secretory leukocyte protease inhibitor (SLPI) is an antimicrobial protein found in saliva and having anti-HIV activity. The concentrations of SLPI in parotid and submandibular/sublingual (SMSL) saliva were determined in an HIV(+) population and compared with uninfected controls. The effect of highly active antiretroviral therapy (HAART) on the concentrations in saliva was determined.

METHODS: Stimulated parotid and SMSL saliva was collected from 65 HIV(+) patients and 19 healthy controls. Flow rates, total protein and SLPI concentrations were determined as well as the effect of HAART on these measurements.

RESULTS: Mean flow rates were reduced for parotid (64%) and SMSL (44%) saliva of HIV(+) patients. Flow rate reductions were unaffected by HAART. Total protein concentration in HIV(+) parotid saliva was increased 56%; patients on HAART had higher concentrations than control. For both groups, SLPI concentrations of SMSL saliva were twice that of parotid saliva. For HIV(+) patients SLPI concentrations of both saliva types were 70% greater than control; the increase in parotid saliva was greater for those taking HAART. For each saliva type, the secretory rate and specific SLPI protein concentration were not different between the groups. Patients with low $CD4^+$ counts had greater SLPI concentrations in parotid saliva than control. There was a negative correlation between $CD4^+$ counts and the SLPI concentration of parotid saliva.

CONCLUSIONS: Salivary flow rate is decreased and the concentration of SLPI is increased in the presence of HIV infection. SLPI concentration in parotid and SMSL saliva is greater with HAART.

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Introduction

Epidemiologic studies suggest that the transmission of the HIV-1 virus in the oral cavity is a rare event (1, 2). Although saliva from HIV-positive individuals may contain viral particles, the amount of infectious viral particles or viral genomes in saliva is usually very low when compared with other body fluids (1, 3, 4). Furthermore, it is well-established that human saliva inhibits HIV infectivity *in vitro* (5–9). Generally, submandibular/sublingual (SMSL) saliva has higher anti-HIV activity than does parotid or whole saliva and unstimulated SMSL has more inhibitory activity than does stimulated SMSL saliva (10). Salivary anti-HIV activity is sensitive to heat and protease treatment, suggesting that the source of the anti-HIV activity in saliva is likely a protein (10).

Secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa acid-stable highly basic non-glycosylated protein (11). Immunohistochemistry indicates that SLPI is secreted by the serous acinar cells of parotid, SMSL and von Ebner glands (11, 12). SLPI has broad antimicrobial activity and has been shown to inhibit candidal (13), bacterial (14) and viral growth (15) *in vitro*. SLPI also plays a role in wound healing (16). It is believed that SLPI contributes to host mucosal defense of oral (7, 12), nasopharyngeal (17), genital (18) and respiratory tissues (19). Depletion of SLPI from saliva results in diminished anti-HIV activity (20) suggesting that this protein is an important component of the anti-HIV activity of human saliva. SLPI has been shown to inhibit HIV-1 infection of human monocytes at physiologic concentrations (1–10 μg/ml) (20). *In vitro* pre-treatment of cells with SLPI protects monocytes against subsequent HIV infection, whereas addition of SLPI to cells after virus exposure has little protective effect (21, 22). This suggests that SLPI exerts its inhibitory effect by blocking HIV binding to host cells (21). A 55 kDa SLPI-binding protein has been found on the surface of human monocytes and it is

proposed that the binding of SLPI to this protein interferes with binding of HIV to host cells (21).

Although salivary anti-HIV activity appears intact in HIV-infected patients (6), a decrease in SLPI concentration in an HIV patient may increase the risk of oral HIV transmission. There are few studies, often conflicting, comparing the concentration of salivary SLPI between HIV-infected patients and healthy subjects. These studies report concentrations of SLPI in unstimulated whole saliva. Two report that the concentrations are similar between HIV patients and healthy controls (8, 23) while a third reports a 10.3% increase when compared with uninfected controls (24).

As the introduction of protease inhibitors in the highly active antiretroviral therapy (HAART) that is currently used to treat HIV(+) patients, there has been a dramatic delay in the progression of disease. There is also a large decrease in oral complications (25). It is unclear whether HAART affects the secretion of SLPI into saliva, which could in turn be a factor for the decrease in oral complications.

This study compared the concentrations of SLPI in glandular saliva (i.e. stimulated parotid and stimulated SMSL saliva) of HIV patients with control subjects. Further, the effect of HAART on the concentrations of SLPI in parotid and SMSL saliva was determined as well as the correlation of SLPI concentrations to $CD4^+$ counts and to viral load.

Material and methods

Subjects

This study is based on 65 HIV(+) male patients with a mean age of 34 who are participants in the US Air Force HIV Natural History Study (26). The purpose of this HIV study is to evaluate disease progression with a standard battery of 30 potential surrogate markers. Clinical manifestations, medication history, viral loads and immune profiles including analysis of T-lymphocyte subsets, immunoglobulins and other blood components are documented in detail. All personnel enrolled in this HIV study are followed twice annually. The majority of these HIV-infected patients are in an early stage of HIV infection, i.e. $CD4^+ > 200$ cells/μl. For a control group, 19 HIV-negative healthy men (defined as HIV-negative, disease-free, not taking medications) of an age range comparable with the HIV(+) group were recruited from the local community. The voluntary, fully informed consent of the subjects used in this research was obtained as required by Air Force Regulation (AFR) 169-9. Approval to undertake this study was obtained from the Institutional Review Boards of both UTHSCSA and Wilford Hall Air Force Medical Center.

To determine the effects of HAART, HIV(+) patients were subdivided into two groups based on whether or not they were undergoing therapy. A patient was considered to be on HAART if he was taking a combination of a protease inhibitor plus two other nucleoside or non-nucleoside reverse transcriptase medications. Of the 65 HIV(+) patients, 31 were undergoing HAART [HAART(+)] and 34 were not [HAART(−)]. For other analyses, the HIV(+) group was also subdivided on the basis of $CD4^+$ counts. Those with $CD4^+$ counts > 200 cells/μl were classified as high $CD4^+$ ($n = 52$) while those having $CD4^+$ counts < 200 cells/μl were classified as low $CD4^+$ ($n = 13$).

Saliva collection

Citrate-stimulated parotid and SMSL saliva was collected according to methods described previously (27). In brief, parotid saliva was collected using a Carlson-Crittenden cup placed over the orifice of Stenson's duct. For collection of stimulated SMSL saliva, the orifices of Wharton's and Bartholin's ducts were isolated with cotton rolls and saliva was collected with gentle suction as it emerged from the ducts (28). Gland stimulation was achieved by swabbing the dorsolateral surfaces of the tongue with a 2% citric acid solution every 30 s. All saliva samples were collected into pre-weighed vials kept on ice and the weight of saliva collected was determined. Flow rate was calculated as the weight of saliva [equal to ml since the specific gravity of saliva is 1 (29)] divided by the collection time to give ml/min (equal to ml/min/gland for parotid saliva). Since SMSL saliva was collected from glands on both right and left sides, the flow rate was divided by 2 to give ml/min/gland. After weighing, the saliva was divided into 100 μl aliquots and stored at −70°C.

Sialochemistry

Total protein in saliva was determined by absorption at 215 nm with bovine serum albumin as a standard (30). An enzyme-linked immunosorbent assay (ELISA) kit was used to determine SLPI concentration in saliva (R&D Systems, Minneapolis, MN, USA). To minimize protein aggregation, ethylendiaminetetraaceitc acid (EDTA) was added to a concentration of 0.9 mM for stimulated parotid saliva and 4.55 mM for stimulated SMSL saliva. For the SLPI assay, saliva was diluted with the buffer provided by the manufacturer.

Statistical analyses

Prior to analyses, all data (except age, $CD4^+$ counts and viral loads) was subjected to square-root transformation. In the Tables and text, $CD4^+$ counts and viral loads are reported as medians with the 25th to 75th percentiles in parentheses. All other data is expressed as the non-transformed mean ± 1 SE.

For analyses of parametric data using two groups [control and HIV(+)], an unpaired *t*-test was used. A P value < 0.05 was considered significant. When the HIV(+) group was subdivided, the parametric data was analyzed with ANOVA [groups = control, HAART(+) and HAART(−) or control, high $CD4^+$ and low $CD4^+$] and where $P < 0.05$, the Bonferroni/Dunn *post hoc* test was used to determine group differences. With this *post hoc* test, $P < 0.0167$ is required for a significant difference between two groups. For viral loads and $CD4^+$ counts, the comparison was between HAART(+) and HAART(−). For these analyses the non-parametric Mann–Whitney *U*-test was used. The possible association between SLPI concentration and

$CD4^+$ counts or between SLPI concentration and viral loads was determined using the Spearman rank correlation analysis.

Results

Demography and HIV status

The mean age of the participants as well as $CD4^+$ counts and viral loads are shown in Table 1. There was no significant difference in age between the control and HIV(+). However, when HIV(+) was subdivided by HAART, the age of HAART(+) was slightly higher than that of HAART(−). The median values for $CD4^+$ number and viral load for the HIV(+) patients were 451 cells/μl and 4504 copies/ml, respectively. Most of the patients were in the early stage of HIV infection with only 13 patients having a $CD4^+$ number <200 cells/μl: six in HAART(−) and seven in HAART(+). There was no difference in $CD4^+$ number between HAART(+) and HAART(−). As might be expected, the viral load of HAART(−) was significantly higher than that of HAART(+) ($P = 0.0007$).

Salivary flow rates

The mean parotid flow rate for HIV(+) was 54% less than that of control ($P < 0.0001$) (Table 2). The mean flow rate of SMSL for HIV(+) was also substantially less (43%) than that of control ($P < 0.0001$). For both HIV(+) HAART subgroups, the flow rates for parotid and for SMSL were significantly lower than the control. There was no difference between HAART(+) and HAART(−) in flow rates of parotid and SMSL saliva.

Total salivary proteins

The mean concentration of total protein in parotid saliva of HIV(+) was significantly higher than that of control (Table 2; $P = 0.0018$). When the HIV(+) group was subdivided by HAART, the protein concentration of HAART(+) was significantly higher than that of control ($P < 0.0001$) and also that of HAART(−) ($P = 0.0097$). There was no difference between control and HAART(−). For SMSL there was no difference in protein concentration between the control and the HIV(+) group or between the control and the HAART subgroups.

Salivary SLPI in HIV infection

The mean concentration of SLPI in parotid saliva of the healthy men was 0.55 μg/ml (Table 3) which is half the 1.13 μg/ml of SMSL saliva (Table 3). For the HIV(+) group, there is a similar proportional difference between SLPI concentrations in parotid and SMSL saliva. When SLPI concentrations were adjusted for flow rate to give SLPI secretory rate (i.e. μg SLPI/min) that for parotid saliva of both control and HIV(+) groups was but half that of SMSL saliva. The specific protein concentration of SLPI (μg SLPI/mg protein) in SMSL saliva of control and of HIV(+) was also more than double that of parotid saliva.

With HIV infection, the concentrations of SLPI were significantly increased (by approximately 70%) in both parotid and SMSL saliva when compared with control (Table 3). However, when adjusted for flow rate, for both types of saliva, there was no significant difference in SLPI secretory rate between control and HIV(+). Also, for both parotid and SMSL saliva, there were no significant differences in specific SLPI protein concentration between control and HIV(+).

Effect of HAART on salivary SLPI

When HIV(+) subjects were subdivided into HAART subgroups based on whether or not the patient was taking HAART, the concentration of SLPI in parotid saliva of HAART(+) was twofold greater than that of control and was also significantly greater (47%) than that of HAART(−) (Table 3). While the mean SLPI concentration for HAART(−) was higher than of control, the mean values for these two groups were not significantly different. For SMSL saliva, SLPI concentration in HAART(+) was also twofold greater than that of control. The SLPI concentration of SMSL for HAART(+) was 44% greater than HAART(−). Similar to parotid saliva, there was no significant difference in SLPI concentration of SMSL saliva between HAART (−) and control groups.

When adjusted for flow rate, for both types of saliva, there was no significant difference in SLPI secretory rate among control and both HAART subgroups. Similarly, for both saliva types, there were no significant differences in specific SLPI protein concentration among control and the two HAART subgroups.

Table 1 Age and HIV status of the control and patient populations[a]

Group	*N*	*Age (years)*	*$CD4^+$ (cells/μl)*	*Viral load (copies/ml)*
Control	19	32.2 ± 1.6	ND[a]	NA[a]
HIV(+)	65	34.6 ± 0.9	451 (259–623)	4504 (400–30 356)
HAART(−)[a]	34	32.2 ± 1.3	532 (300–670)	12 454 (2228–37 445)
HAART(+)[a]	31	36.9 ± 1.1[b]	407 (227–554)	440 (400–9258)[c]

[a]Values for age are mean ± 1 SE; values for $CD4^+$ and viral load are median with the 25th to 75th percentiles given in parentheses. ND, not determined; NA, not applicable; HAART(−), HIV(+) patients who are not taking highly active antiretroviral therapy (HAART); HAART(+), HIV(+) patients receiving HAART.
[b]$P < 0.01$ when compared with HAART(−), Bonferroni/Dunn *post hoc* test.
[c]$P < 0.001$ when compared with HAART(−), Mann–Whitney *U*-test.

Table 2 Flow rates and total protein concentration of stimulated parotid and stimulated submandibular/sublingual (SMSL) saliva for the control and the patient populations[a]

	Flow rate (ml/min/gland)		*Total protein (mg/ml)*	
Group	*Parotid*	*SMSL*	*Parotid*	*SMSL*
Control	0.56 ± 0.06 ($n = 19$)	0.53 ± 0.03 ($n = 19$)	2.45 ± 0.22 ($n = 19$)	2.16 ± 0.13 ($n = 19$)
HIV(+)	0.26 ± 0.03[b] ($n = 65$)	0.30 ± 0.02[b] ($n = 65$)	3.76 ± 0.24[c] ($n = 51$)	2.42 ± 0.11 ($n = 64$)
HAART(−)	0.28 ± 0.03[d] ($n = 34$)	0.31 ± 0.03[e] ($n = 34$)	3.25 ± 0.34 ($n = 26$)	2.26 ± 0.12 ($n = 34$)
HAART(+)	0.25 ± 0.04[e] ($n = 31$)	0.29 ± 0.03[e] ($n = 31$)	4.28 ± 0.31[e,f] ($n = 25$)	2.61 ± 0.18 ($n = 30$)

[a]HAART(−), HIV(+) patients who do not take highly active antiretroviral therapy (HAART); HAART(+), HIV(+) patients receiving HAART. Values in the Table indicate the mean ± 1 SE. The number of individuals assayed is given in parentheses in each cell.
[b]$P < 0.0001$ when compared with the control group, unpaired *t*-test.
[c]$P < 0.002$ when compared with the control group, unpaired *t*-test.
[d]$P < 0.0002$ when compared with the control group, Bonferroni/Dunn *post hoc* test.
[e]$P < 0.0001$ when compared with the control group, Bonferroni/Dunn *post hoc* test.
[f]$P < 0.01$ when compared with HAART(−), Bonferroni/Dunn *post hoc* test.

Table 3 Secretory leukocyte protease inhibitor (SLPI) concentration, secretory rate and specific protein concentration for stimulated parotid saliva and stimulated submandibular/sublingual saliva (SMSL) for the control and patient populations[a]

		HIV(+)		
	Control	*All*	*HAART(−)*	*HAART(+)*
N (parotid/SMSL)	19/19	51/64	26/34	25/30
Concentration (μg/ml)				
Parotid	0.55 ± 0.08	0.94 ± 0.11[b]	0.76 ± 0.16	1.12 ± 0.13[c,d]
SMSL	1.13 ± 0.16	1.91 ± 0.20[b]	1.63 ± 0.24	2.23 ± 0.33[d,e]
Secretory rate (μg/min)				
Parotid	0.31 ± 0.05	0.25 ± 0.03	0.24 ± 0.05	0.26 ± 0.03
SMSL	0.58 ± 0.07	0.55 ± 0.08	0.47 ± 0.07	0.63 ± 0.15
Specific concentration (μg/mg protein)				
Parotid	0.23 ± 0.03	0.26 ± 0.02	0.23 ± 0.03	0.29 ± 0.04
SMSL	0.56 ± 0.10	0.84 ± 0.09	0.76 ± 0.11	0.93 ± 0.15

[a]HAART(+), HIV(+) patients receiving highly active antiretroviral therapy (HAART); HAART(−), HIV(+) patients who do not take active antiretroviral therapy (HAART). Except for *N*, the values in the Table indicate the mean ± 1 SE.
[b]$P < 0.05$ when compared with control, unpaired *t*-test.
[c]$P < 0.002$ when compared with the control group, Bonferroni/Dunn *post hoc* test.
[d]$P < 0.02$ when compared with HAART(−), Bonferroni/Dunn *post hoc* test.
[e]$P < 0.02$ when compared with the control group, Bonferroni/Dunn *post hoc* test.

Effect of $CD4^+$ counts on salivary SLPI

When HIV(+) was subdivided by $CD4^+$ counts, the mean concentration of SLPI in parotid saliva for low $CD4^+$ (<200 cell/μl) was double, and significantly ($P = 0.0126$) greater than that of control (Table 4). The SLPI concentration in parotid saliva of high $CD4^+$ (≥200 cells/μl) was midway between control and low $CD4^+$ and was not significantly different from either group. Although the mean concentration of SLPI in SMSL saliva of low $CD4^+$ was 85% greater than that of control, there was no significant difference among the groups in SLPI concentrations. For each saliva type, there were no significant differences among the three groups in SLPI secretory rate and SLPI-specific protein concentration (data not shown). There was a negative correlation for SLPI concentration in parotid saliva and $CD4^+$ counts (Spearman rank correlation: $P = 0.038$). There was no significant correlation for SLPI in SMSL and $CD4^+$ counts. There was no correlation between viral load and SLPI concentrations either in parotid or SMSL saliva.

Discussion

In this study, salivary SLPI concentration, secretory rate and specific protein concentration was determined in parotid and SMSL saliva of both HIV uninfected and infected individuals. The concentration of SLPI in both parotid and SMSL saliva is increased in HIV(+) individuals. These findings are consistent with a previous report that the SLPI concentration in unstimulated whole saliva of HIV(+) patients is higher in comparison with uninfected controls (24). While the increase in salivary SLPI may be due to a decrease in salivary flow rates in HIV(+) patients, in this study, at least for parotid saliva, the concentration of SLPI was negatively correlated with $CD4^+$ counts. In addition, HIV(+) patients receiving HAART had greater concentrations of SLPI in parotid saliva than did HIV(+) patients not receiving HAART.

The reason for the increase in SLPI concentrations with HIV-infection is not known. In the present study, when SLPI is expressed as secretion rate (μg/min) or in terms of specific protein concentration (μg SLPI/mg

Table 4 The concentration of secretory leukocyte protease inhibitor (SLPI) in stimulated parotid and stimulated submandibular/sublingual (SMSL) saliva of control and HIV(+) patients, the latter grouped according to high $CD4^+$ (>200 cells/μl) and low $CD4^+$ (<200 cells/μl) counts

	Parotid		*SMSL*	
Group	*n*	*μg/ml*	*n*	*μg/ml*
Control	19	0.55 ± 0.08	19	1.13 ± 0.16
HIV(+)				
Low $CD4^+$	10	1.16 ± 0.23[a]	13	2.09 ± 0.52
High $CD4^+$	41	0.88 ± 0.12	51	1.86 ± 0.22

The concentration is mean ± 1 SE.
[a]$P = 0.0126$ when compared with the control group (Bonferroni/Dunn *post hoc* test.)

total protein), there are no significant differences for either saliva type between control and HIV(+) patients. These results suggest that the increase in SLPI concentration in HIV patients may not be due to an alteration in the synthesis or secretion of SLPI, but rather than the increased salivary concentration is the result of decreased fluid secretion. Factors that may contribute to decreased fluid secretion include taking xerostomic medications, gland inflammation, smoking habits, etc. Based on our earlier study, it is unlikely that the decreased fluid secretion in the HIV(+) patients results from taking xerostomic medications. In that study, we showed that there was no difference in the flow rates between HIV patients who took xerostomic medications when compared with those that did not (31). The smoking habits of the patients and controls and the extent of gland inflammation were not determined in this study. However, previous studies suggest that the concentration of SLPI is increased with inflammation (32, 33) and there are studies suggesting an inflammatory response in salivary glands with HIV infection (34). Therefore, the increase SLPI in the HIV(+) group may also be indicative of an inflammatory condition in the salivary glands. A recent study suggested that smoking might cause a decrease in salivary flow rates (35). As the smoking habits of the patients and controls in this study were not monitored, it is unclear whether smoking could play a role in the reduced flow rate and increased SLPI concentrations shown in this study.

As the introduction of protease inhibitors in HAART, the progression of HIV disease and the occurrence of oral complications have dramatically decreased (25). This study shows that salivary SLPI concentrations in both stimulated parotid and stimulated SMSL saliva for HIV(+) patients receiving HAART are double that of the uninfected control. For parotid saliva, the SLPI concentration for those on HAART is almost 50% greater than those not on HAART. Although not statistically significant, the directions of the changes for concentration of SLPI in SMSL saliva are similar to those of parotid saliva. Compared with parotid saliva, SLPI concentration, secretory rate and specific protein concentration are twofold greater in SMSL saliva. The clinical benefits of HAART are, in general, to suppress the viral load and to enhance the host immune system (36, 37). Although a lower serum viral load is evident in HAART(+) patients when compared with HIV(+) patients who were not under HAART, the increase in salivary SLPI concentration was not associated with the serum viral load in our study. In contrast to this study, a tendency for a lower salivary SLPI concentration in HIV(+) patients using protease inhibitors and lower viral load has been reported previously (24). Whether this discrepancy is due to cohort effect, sample size, saliva types (glandular saliva in this study vs. whole saliva used in other studies) or to experimental design needs clarification. However, in addition to the reported direct antimicrobial effect of HAART on oral infection (38–42), our study suggests that HAART may also enhance SLPI concentration in saliva. Thus, as salivary SLPI concentration is increased with HAART, in addition to its other beneficial effects, may also, through its effect on SLPI, reduce the risk of oral transmission of HIV.

Current results also indicate that SLPI concentrations in parotid saliva are higher in HIV(+) patients with low $CD4^+$ counts than in patients with high $CD4^+$ counts. A similar trend is also observed in SMSL saliva. Furthermore, parotid SLPI concentration has a negative correlation with $CD4^+$ counts. At the present time, it is unknown whether the increased salivary SLPI concentration of patients with low $CD4^+$ counts is indicative of a more advanced stage of HIV disease. Clarification of this requires further studies with larger sample sizes.

One observation of note is that the concentration of SLPI as well as its secretory rate and specific protein concentration in stimulated SMSL saliva is almost double that of parotid saliva. This could be important since, during both stimulated and unstimulated salivary gland secretion, SMSL saliva contributes more than 50% of the total saliva volume (43). The concentration of SLPI in stimulated SMSL saliva is within the range shown to inhibit HIV-1 infection of human monocytes (1–10 μg/ml) (20). Other studies suggest that the anti-HIV activity is higher in SMSL saliva than in whole saliva or in parotid saliva (10). The present study showing a greater concentration of SLPI in SMSL saliva may, at least in part, account for the higher anti-HIV activity of SMSL saliva.

In summary, the flow rates of stimulated parotid and stimulated SMSL saliva are significantly reduced in this cohort of HIV patients and the concentration of SLPI in these secretions is increased when compared with that of controls. The higher concentration of SLPI in SMSL saliva when compared with that of parotid saliva may account for the higher anti-HIV-1 activity of SMSL

saliva (10). This study indicates that the salivary anti-HIV factor, SLPI, is not only preserved in HIV infection but that HAART may enhance the salivary concentration of SLPI which in turn may further reduce the risk of oral transmission of HIV.

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