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ORIGINAL ARTICLE

Expression of secretory leukocyte proteinase inhibitor in the submandibular glands of AIDS patients

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OBJECTIVE: Secretory leukocyte proteinase inhibitor (SLPI) is an endogenous proteinase inhibitor present in mucosal secretions. It also displays antimicrobial activity including anti-human immunodeficiency virus activity. This protease inhibitor is also expressed in submandibular glands (SMG), but there are few data on its expression in AIDS patients with infectious conditions.

METHODS: We analyzed the expression of SLPI using immunohistochemistry in submandibular gland samples of 36 AIDS patients [10 with normal histology, 10 with chronic nonspecific sialadenitis, eight with mycobacteriosis, and eight with cytomegalovirus (CMV) infection] and 10 HIV-negative controls. The proteinase inhibitor was quantified using image analysis and expressed as % of positively stained area.

RESULTS: There was a higher expression of SLPI in AIDS patients with CMV infection (% of stained area, mean ± SD: 37.37 ± 14.45) when compared with all other groups ($P = 0.009$). There were no significant differences between control subjects (22.70 ± 9.42%) and AIDS patients without histologic alterations (18.10 ± 7.58%), with chronic nonspecific sialadenitis (17.13 ± 5.36%), or mycobacterial infection (21.09 ± 4.66%).

CONCLUSION: Cytomegalovirus infection increases SLPI expression in the SMG of AIDS patients. Our results reveal new insights into the pathogenic association between HIV and CMV in AIDS patients.
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Keywords: secretory leukocyte proteinase inhibitor; submandibular gland; AIDS; immunohistochemistry; cytomegalovirus; autopsy

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Introduction

The oral mucosa is exposed to high concentrations of microorganisms and relies on an effective innate immune system that provides host defense against infection. In particular, oral tissues are naturally resistant to the human immunodeficiency virus type 1 (HIV-1) infection. A series of secreted antimicrobial factors present in the oral fluids have been implicated in this resistance, and include secretory leukocyte proteinase inhibitor (SLPI), defensins, salivary agglutinin, and thrombospondin (Shugars and Wahl, 1998; Shugars *et al*, 1999).

Secretory leukocyte proteinase inhibitor is a cationic serine protease inhibitor present in large quantities in mucosal secretions including saliva. Originally, it was identified as an inhibitor of neutrophil elastase, but subsequent studies revealed broad-spectrum antimicrobial activities, anti-inflammatory activities, and involvement in wound repair (Hiemstra, 2002; Sallenave, 2002; Doumas *et al*, 2005). Several studies have shown that SLPI displays marked anti-HIV-1 activity both *in vivo* and *in vitro* (McNeely *et al*, 1995; Shugars *et al*, 1997; Wahl *et al*, 1997b). This activity is thought to result from blocking an early step in infection that occurs prior to virus internalization and reverse transcription, as demonstrated by binding of SLPI to the HIV-1 co-factor annexin II (Shugars and Wahl, 1998; Shugars *et al*, 1999; Ma *et al*, 2004). Besides its anti-HIV-1 inhibitory action, SLPI has also been shown to possess antibacterial and antifungal properties (Hiemstra *et al*, 2004).

In oral tissues, expression of SLPI has been demonstrated in keratinocytes, and in the major and minor salivary glands (Ohlsson *et al*, 1984; Jana *et al*, 2005). Lin *et al* (2004) demonstrated that SLPI concentrations in submandibular/sublingual and parotid saliva from HIV-infected individuals are higher than those in healthy subjects.

In patients with advanced AIDS, the presence of inflammatory, infectious, and neoplastic diseases in the oral cavity is frequent. We have previously demonstrated the presence of inflammatory, infectious, and neoplastic abnormalities in 51% of the parotid glands

of the patients who died from AIDS in Brazil, and showed that mycobacteriosis and cytomegalovirus (CMV) were the most common infections (Vargas *et al*, 2003). Although the role of SLPI in inhibiting HIV infection in the oral cavity has been documented, few studies analyzed the association of SLPI with infectious conditions in the oral cavity that are associated with HIV infection (Wahl *et al*, 1997a; Shugars *et al*, 1999). Chattopadhyay *et al* (2004) identified high salivary SLPI levels as one of the risk factors for the development of oralpharyngeal candidiasis in HIV-1-infected subjects. Therefore, the objective of this study was to analyze the expression of SLPI in the submandibular glands (SMG) of patients who died from AIDS and relate these findings to the presence of infectious and inflammatory processes.

Methods

The Ethics Committees of the Sao Paulo University Medical School and the Faculty of Odontology of Piracicaba, University of Campinas approved the use of autopsy samples for the present study.

Patient population
We analyzed SMG samples of 36 patients with AIDS and 10 HIV-1-negative patients who had died in the University Hospital of the São Paulo Medical School between 1996 and 1999 and were autopsied in the Department of Pathology of the Faculty of Medicine of the University of São Paulo.

A complete autopsy was performed in all patients. Clinical records were reviewed in order to obtain age, sex, and CD4 cell count. Final autopsy reports were analyzed for main diseases. We have previously described tongue and parotid histologic characteristics of this population (Vargas *et al*, 2003; de Faria *et al*, 2005).

Tissue processing and histologic analyses
Submandibular gland samples were fixed in buffered 10% formalin solution for 24 h, and routinely processed; two to six sections of each patient were available for analysis. Slides were stained with H&E, Ziehl-Neelsen and Grocott stainings. Four histologic categories were analyzed: samples without histologic abnormalities, chronic nonspecific sialadenitis, mycobacterial infection, and CMV infection. Mycobacterial infection was determined by the presence of poorly formed granulomas with necrosis and presence of positive bacilli identified by Ziehl-Neelsen staining. CMV infection was defined by the presence of typical enlarged cells with large nuclei with a prominent eosinophilic nucleolus and clear nuclear halo, present mainly in ductal cells and in some serous acinar cells, associated with different degrees of chronic inflammation (Vargas *et al*, 2003). Chronic nonspecific sialadenits was defined by the presence of variable degrees of lymphocytic infiltration in the gland parenchyma without identification of etiologic agents by the techniques cited (Vargas *et al*, 2003). No microbiological studies were performed in the samples.

Immunohistochemistry
The expression of SLPI in tissue was revealed by immunohistochemistry using a monoclonal anti-SLPI antibody (clone 31) essentially as described (Aarbiou *et al*, 2004). For the detection of the major-core protein p24 in the samples of the AIDS patients, the primary monoclonal antibody anti p24 (clone Kal-1; DAKO, Glostrup, Denmark) was used. In short, the sections were incubated overnight with the primary antibody at 4°C following antigen retrieval using citrate buffer. As a secondary antibody the horseradish peroxidase conjugated anti-mouse EnVision system (DAKO) was used, with diaminobenzidine (Sigma Chemical Co., St Louis, MO, USA) as a chromogen. The sections were counterstained with Mayer's hematoxylin. For negative controls, the primary antibodies were omitted from the procedures.

Morphometry
The percentage of SLPI-stained acinar gland area was determined by using image analysis. Measurements were performed with the software Image-Pro® Plus 4.1 for Windows® (Media Cybernetics, Silver Spring, MD, USA) on a personal computer connected to a digital camera (JVC TK-C1380 color video camera; Victor Company of Japan, Yokohama, Japan) that was coupled to a light microscope (Leica DMR; Leica Microsystems Wetzlar Gmbh, Wetzlar, Germany). We measured the area of positive SLPI staining in at least 10 randomly selected acinar areas of the glands at 200× magnification. Selection of areas to be analyzed was performed by an investigator unaware of the study group. SLPI was expressed as % of stained area ($\mu m^2/\mu m^2$).

Statistical analysis
Statistical analysis was performed with the SPSS 13.0 software (SPSS, Chicago, IL, USA). A multiple comparison using one-way analysis of variance (ANOVA) followed by Tukey's test was applied for comparison of SLPI expression among the different groups. To analyze the correlation between SLPI expression and CD4 counts, the Spearman test was applied. Results were expressed as mean ± standard deviation (SD). The level of significance was set at $P < 0.05$.

Results

Patient population
In the control group there were six males and four females. Mean age was 58.4 years, ranging from 32 to 77 years. None of the patients presented salivary gland-related complaints, gland enlargement or medical suspicion of submandibular disease prior to death. Autopsy findings of the control group are presented in Table 1.

In the AIDS group there were 25 males and 11 females. Ages ranged from 24 to 65 years, with a mean of 37.7 years. The last CD4 levels prior to death were obtained in 21 patients, with a median of 90 (ranging from 5 to 434) cells μl^{-1}, with 86% of the patients presenting < 200 cells μl^{-1}. None of the patients

Table 1 Sex, age, and main autopsy findings of the HIV-negative control group

Patient	*Age (years)*	*Sex*	*Autopsy findings*
1	63	Female	Chagas' disease, atherosclerosis
2	67	Male	Pneumonia
3	61	Male	Atherosclerosis, diabetes mellitus
4	77	Male	Cerebral aneurysm
5	32	Female	Cerebral aneurysm
6	57	Male	Ischemic cerebral stroke
7	77	Female	Hemorrhagic cerebral stroke
8	56	Male	Hemorrhagic cerebral stroke
9	45	Male	Renal transplantation with gastrointestinal bleeding
10	49	Female	Myasthenia gravis, acute pancreatitis

presented salivary gland-related complaints, gland enlargement, or medical suspicion of submandibular gland disease prior to death (Table 2).

Histologic characterization

In the control group no histologic alterations were found in the SMG (Figure 1a). In the AIDS group, 10 patients presented no histologic alterations, 10 had chronic nonspecific sialadenitis (Figure 1b), eight had mycobacteriosis (Figure 1c), and eight had CMV infection in the SMG (Figure 1d).

Immunohistochemical findings

The expression of SLPI was present in serous acinar cells but not in mucosal acinar cells, gland stroma, or ductal cells, with a granular staining pattern (Figure 2a,d,e). There was no SLPI expression in the granulomas or in inflammatory cells (Figure 2b). Especially in the cases with extensive CMV infection in the gland, SLPI expression was also observed in some of the CMV-infected ductal cells (Figure 2c).

There was no p24 expression in the acinar or ductal gland areas of all cases examined. The presence of antigen was observed in the histiocytes of a single case with mycobacterial infection.

Morphometrical analysis

There was no significant difference in SLPI expression in acinar areas among control subjects [% SLPI stained area (mean ± SD): 22.70 ± 9.42] and AIDS patients without histologic alterations in SMG (18.10 ± 7.58%, $P = 0.77$), with chronic nonspecific inflammation

Table 2 Age, sex, CD4 cell counts, and main autopsy findings of the AIDS patients

Patient	*Age (years)*	*Sex*	*CD4 count (cells μl^{-1})*	*Submandibular gland alterations*	*Autopsy findings*
11	45	Female	26	No	Cachexia, CMV gastritis
12	47	Male	13	No	Bronchopneumonia
13	41	Male	41	No	Bronchopneumonia
14	40	Male	71	No	CMV pneumonia, *Cryptococcus* meningitis
15	32	Male	NA	No	Granulomatous disease, cachexia
16	33	Male	05	No	Pneumocystosis, chronic pancreatitis
17	30	Male	22	No	Disseminated tuberculosis
18	30	Male	67	No	Lung mycobacteriosis, neurotoxoplasmosis
19	31	Male	NA	No	Cachexia, acute respiratory failure
20	34	Male	NA	No	Disseminated histoplamosis
21	45	Male	NA	Sialadenitis	Disseminated tuberculosis
22	29	Female	NA	Sialadenitis	Disseminated mycobacteriosis, septic shock
23	65	Male	93	Sialadenitis	Pleural tuberculosis
24	50	Female	NA	Sialadenitis	Septic shock
25	40	Male	375	Sialadenitis	Bronchopneumonia, meningitis
26	46	Male	290	Sialadenitis	Neurocryptococosis, bronchopneumonia
27	36	Female	31	Sialadenitis	Pneumocystosis
28	38	Male	434	Sialadenitis	Disseminated histoplamosis, septic shock
29	26	Male	72	Sialadenitis	Interstitial pneumonia, ganglionic tuberculosis
30	44	Male	66	Sialadenitis	Disseminated NHL, esophagic candidiasis
31	46	Male	50	Mycobacteriosis	Disseminated mycobacteriosis
32	34	Female	NA	Mycobacteriosis	Disseminated mycobacteriosis
33	40	Female	NA	Mycobacteriosis	Disseminated mycobacteriosis, cachexia
34	24	Male	NA	Mycobacteriosis	Disseminated mycobacteriosis
35	48	Female	NA	Mycobacteriosis	Pneumonia associated with HIV
36	35	Male	NA	Mycobacteriosis	Pneumocystosis
37	43	Male	NA	Mycobacteriosis	Disseminated mycobacteriosis
38	29	Male	36	Mycobacteriosis	Septic shock, disseminated mycobacteriosis
39	30	Female	05	CMV	Esophagic candidiasis, disseminated mycobacteriosis, CMV
40	37	Female	NA	CMV	Bacterial bronchopneumonia, lung and adrenal CMV infection
41	26	Female	NA	CMV	Neurotoxoplasmosis, lung and adrenal CMV infection
42	32	Male	47	CMV	Septic shock, CMV, genital herpes
43	26	Male	15	CMV	CMV and mycobacteriosis, cachexia
44	50	Male	NA	CMV	Bacterial bronchopneumonia, cachexia, CMV
45	51	Male	136	CMV	Cachexia, CMV
46	25	Female	10	CMV	Neurotoxoplasmosis, CMV

CMV, cytomegalovirus; NA, not available; NHL, non-Hodgkin lymphoma.

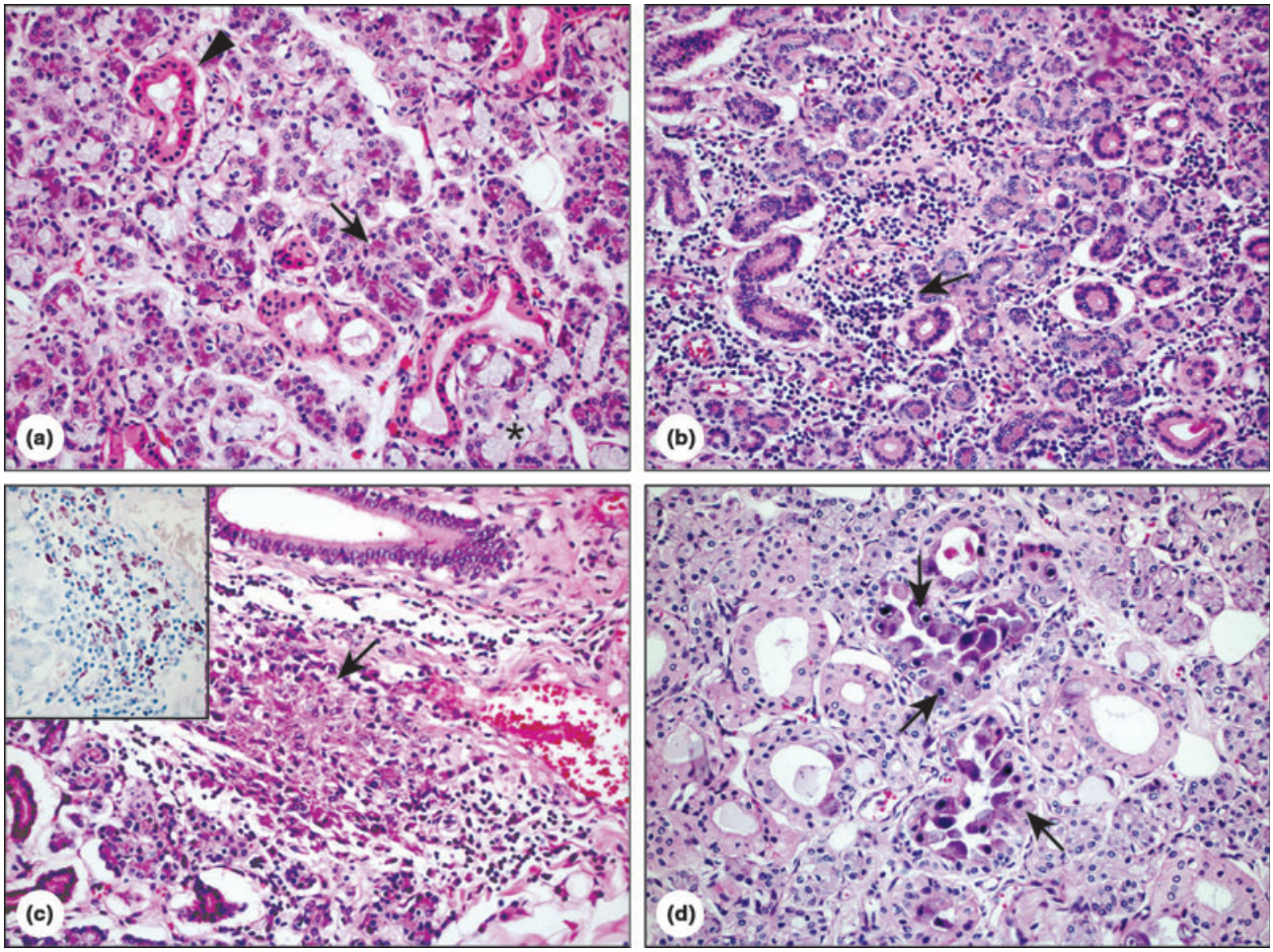

Figure 1 Histologic features of submandibular glands in AIDS. (**a**) Submandibular gland without histologic alterations in a control patient. Serous acinus (arrow), mucous acinus (asterisk), ductal cells (arrowhead; 200×, H&E). (**b**) Chronic sialadenitis in an AIDS patient. Observe the chronic inflammation (arrow) surrounding glandular structures (200×, H&E). (**c**) Mycobacterial infection in the submandibular gland of an AIDS patient. Observe the poorly formed granuloma (arrow; 200×, H&E). In the insert, multiple acid-fast bacilli (400×, Ziehl-Neelsen). (**d**) CMV infection in the submandibular gland of an AIDS patient. The ductal cells show the typical large intranuclear inclusions (arrow; 200×, H&E)

(17.13 ± 5.36%, $P = 0.62$) or with mycobacterium infection (21.09 ± 4.66%, $P = 0.99$). However, there was a statistically significant higher expression of SLPI in patients with CMV-infected SMG (% SLPI = 37.37 ± 14.45) when compared with all other groups ($P = 0.009$) (Figure 3). The inverse correlation between percentage of SLPI and CD4 cells counts did not reach statistical significance ($r^2 = -0.22$, $P = 0.33$).

Discussion

In the present study, we analyzed tissue expression of SLPI in the SMG of patients who died from advanced AIDS, and demonstrated a higher SLPI expression in CMV-infected SMG. No difference was noted in SLPI expression in SMG from AIDS and control subjects. These results suggest that CMV infection increases local expression of SLPI in SMG in AIDS.

There are only few studies that have quantified tissue expression of SLPI in patients with inflammatory and infectious conditions related to AIDS and, to the best of our knowledge, we are the first to detect a higher SLPI expression in patients with CMV infection (Wahl *et al*, 1997b). SLPI is an endogenous proteinase inhibitor that has two highly homologous domains of about 54 amino acids, each of which contains four disulfide bonds. It was isolated from a variety of mucosal secretions, including human parotid fluids (Thompson and Ohlsson, 1986). It was identified based on its ability to inhibit neutrophil elastase, and subsequently shown to inhibit a range of proteinases from inflammatory cells (Doumas *et al*, 2005). In addition, SLPI displays anti-inflammatory and antimicrobial activities, and is implicated in epithelial wound repair (Hiemstra, 2002; Sallenave, 2002; Doumas *et al*, 2005). Particularly, SLPI plays an important role in defense against HIV-1 infection, especially in the early stages of infection (McNeely *et al*, 1997).

Tissue expression of SLPI in the salivary glands has been previously observed. Ohlsson *et al* (1984) described SLPI expression in serous cells of both parotid and SMG. Wahl *et al* (1997b) analyzed oral tissue expression of SLPI in HIV-positive (including 10 patients with a CMV infection) and HIV-negative tissue samples, and did not

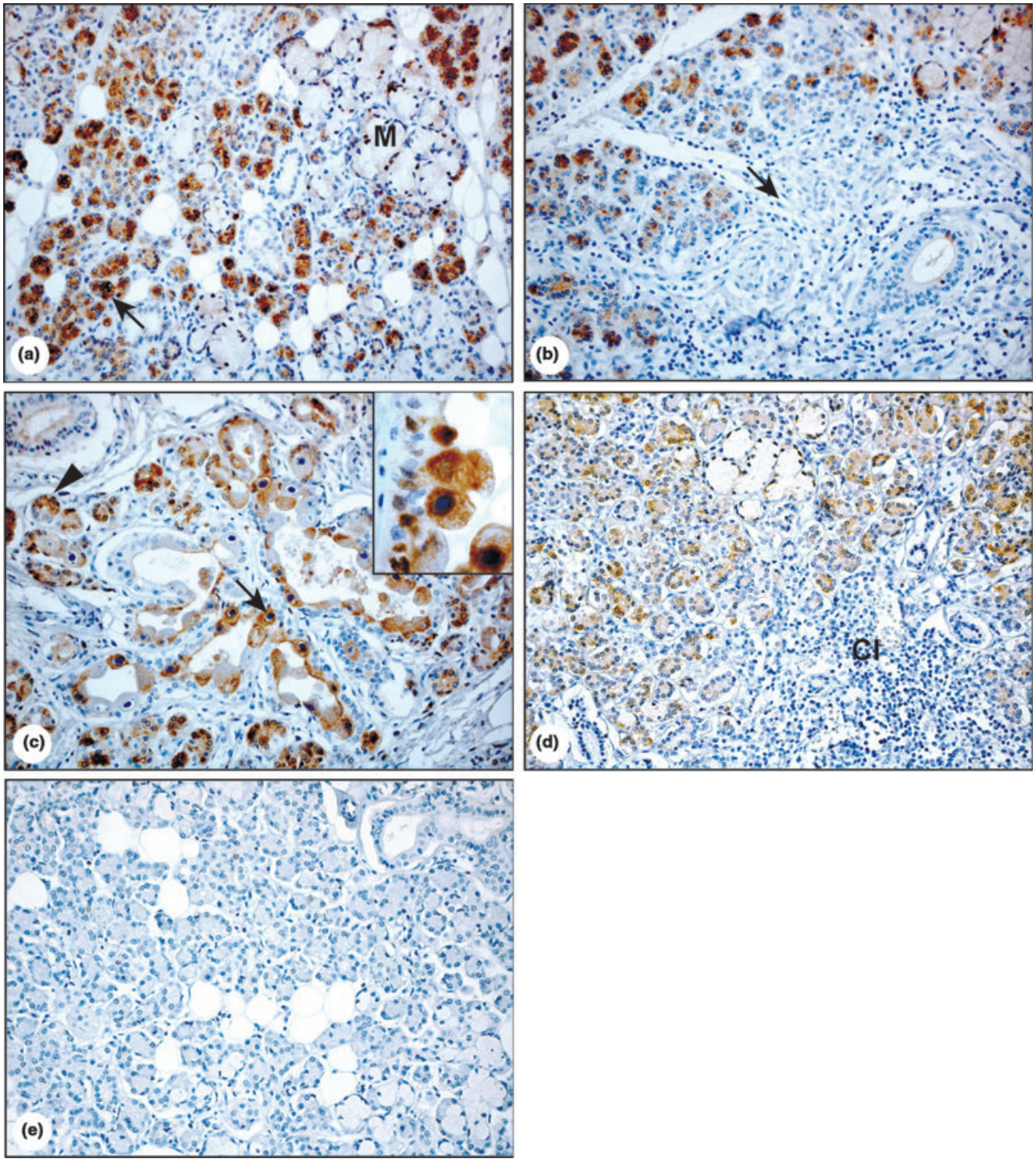

Figure 2 Immunostaining for SLPI in submandibular glands. (**a**) SLPI immunohistochemical staining in a submandibular gland without histologic alterations. SLPI staining is present in serous acinar cells (arrow) but absent in mucous cells (M; 200×). (**b**) SLPI immunohistochemical staining in a submandibular gland with mycobacteriosis. Note the absence of staining in the granuloma formation (arrow; 200×). (**c**) SLPI immunohistochemical staining in a submandibular gland with CMV infection. Serous acinar cells (arrowhead) and ductal cells (arrow) present strong positive staining (200×). In the insert, a granular SLPI staining pattern in ductal cells infected by CMV (1000×). (**d**) SLPI immunohistochemical staining in a submandibular gland with chronic sialadenitis (CI) in an AIDS patient (200×). (**e**) SLPI immunohistochemical negative control in the submandibular gland of a control patient (200×)

detect qualitative differences in expression between the groups. Our results confirm their descriptive findings. Similarly, we were not able to detect differences in SLPI between control groups and those AIDS patients without an HIV-related infectious condition, but detected increased expression in CMV-infected glands.

More recently, Lin *et al* (2004) studied the salivary SLPI concentration, secretory rate, and specific protein

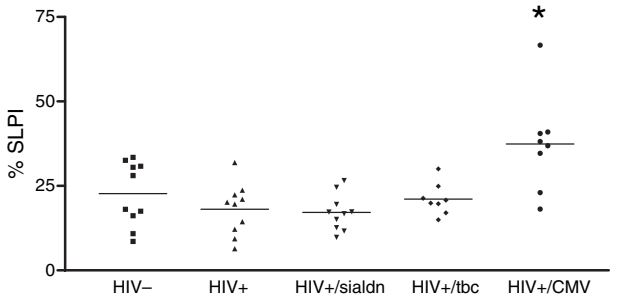

Figure 3 Mean values of the % SLPI staining in the submandibular glands (SMG) of the different study groups. (*) There was a significant increase in the CMV-infected group when compared with all other study groups ($P = 0.009$). The horizontal line represents the mean. SLPI, secretory leukocyte proteinase inhibitor; HIV, human immunodeficiency virus, sialdn, sialadenitis; tbc, tuberculosis; CMV, cytomegalovirus

concentration in parotid and submandibular/sublingual secretions of HIV-1-infected and -uninfected individuals. The concentration of SLPI was increased in the infected group and most notably, mainly in HIV-1-infected individuals who received highly active antiretroviral therapy (HAART). The authors suggested that this increase in infected patients results from decreased fluid secretion rather than from an alteration in the synthesis or secretion of SLPI *per se*. Gordon *et al* (2005) could not demonstrate differences in SLPI levels in the bronchoalveolar lavage fluid of HIV-positive patients and suggested an altered innate pulmonary immunity in these patients.

Even in the era of HAART therapy, CMV is still an important opportunistic infection in HIV patients, with evidence that CMV may have direct and indirect effects on HIV pathogenicity (Steininger *et al*, 2006). In this study, we have demonstrated that the expression of SLPI is increased in the SMG of AIDS patients with a CMV infection, suggesting a role of SLPI in CMV-infected HIV patients. SLPI is synthesized and secreted at mucosal sites in response to microbial exposure, inflammation, injury and repair, and its epithelial expression was found to be increased by e.g. neutrophil defensins, growth factors, and pro-inflammatory cytokines, being possibly part of a local inducible defense system. Previous studies have shown that, in addition to its antibacterial and antifungal activities, SLPI also displays antiviral activity. It is not only active against HIV-1, but also against Sendai virus and influenza A virus (Beppu *et al*, 1997). The anti-CMV activity of SLPI is not well documented and therefore the contribution of increased SLPI expression to anti-CMV defenses remains to be established. In addition to its antimicrobial activity, SLPI may either provide protection against proteinases derived from inflammatory cells or it may contribute to repair of oral mucosa secondary to CMV-induced tissue injury (Wahl *et al*, 1997b). The recent observation that mice deficient in SLPI have impaired oral wound healing is in line with this suggestion (Angelov *et al*, 2004). The mechanisms by which CMV increases SLPI expression are unknown. The recent observation that CMV causes activation of the epidermal growth factor receptor (EGFR) combined with the observation that the EGFR ligand transforming growth factor-beta (TGFβ) increases epithelial SLPI expression suggests that CMV may employ EGFR to increase SLPI expression (Sorensen *et al*, 2003; Wang *et al*, 2003). This possibility is currently explored.

Although SLPI expression in SMG with mycobacteriosis was higher than in the group without opportunistic infectious, the differences were not statistically significant. It has been previously demonstrated that SLPI expression can be increased in rat macrophages exposed to *Mycobacterium tuberculosis*, but further data on humans are not available (Ding *et al*, 2005).

There was no p24 expression in most of our samples, so we could not check whether the local presence of the virus core protein in the SMG could alter SLPI content expression. In line with other authors (Bruner *et al*, 1989; Elliott and Oertel, 1990; Wahl *et al*, 1997b), we were not able to detect p24 protein expression in gland parenchyma. It is possible that more sensitive methods are needed to detect HIV in oral tissue samples, as Wahl *et al* (1997a) detected HIV RNA by *in situ* hybridization in interstitial mononuclear cells, but not in epithelial cells, in more than 30% of the 55 infected salivary glands in their study. The authors suggested that the distinct anatomic distribution between SLPI expression in the gland epithelial cells and HIV RNA in stromal mononuclear cells appears to partition the virus from the inhibitor.

Our study has some limitations. In many of the cases, we did not have access to information on the anti-HIV-1 treatment these patients received. This may be relevant, because it has been recently shown that HAART therapy may enhance SLPI concentration in saliva (Lin *et al*, 2004). As our samples date back to 1996–1999, it is possible that not all patients were receiving HAART at that time. Our groups do not include CMV+/HIV− patients, so we cannot exclude that increased SLPI expression is specific for HIV-infected patients with CMV infection or CMV infection alone. However, it is extremely difficult to obtain tissue containing an adequate number of CMV-infected salivary glands in non-HIV patients at autopsy. Although immunohistochemistry is not an analytical method that allows exact quantification of the amount of SLPI expression, we did collect important information on the relative amount of SLPI expression because this expression was analyzed as percentage stained area. This outcome measure does allow a relative comparison of expression levels between the two patient groups studied in the present report.

Although not statistically significant, there was a negative correlation between CD4 cells counts and SLPI ($r^2 = -0.22$) in our patients. Lin *et al* (2004) also recently described an inverse correlation between SLPI levels in the parotid gland saliva and CD4 counts, and hypothesized that increased SLPI levels in the saliva are indicative of a more advanced stage of the HIV disease. In our samples, most of the patients were profoundly immunosuppressed as indicated by the very low CD4

cell counts, which could explain the lack of significance in the correlation analysis.

In summary, we have shown that the presence of CMV infection in SMG of AIDS patients is associated with an increased local expression of SLPI protein. This increased SLPI expression may be associated with different events related to CMV-induced HIV infection in oral tissues such as protection against infection, the action of proteinases derived from inflammatory cells and the promotion of tissue repair (Griffiths, 2006). Our results further reinforce the evidence of a possible CMV/HIV pathogenic interaction in AIDS patients (Steininger *et al*, 2006).

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