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Matrix metalloproteinase-7, -8, -9, -25, and -26 and CD43, -45, and -68 cell-markers in HIV-infected patients' saliva and gingival tissue

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BACKGROUND: Matrix metalloproteinases (MMPs) process the extracellular matrix and act in tissue remodelling in many physiological and pathological conditions. Certain MMPs can also exert protective anti-inflammatory properties. The levels and expression of MMPs and tissue inhibitors of MMPs (TIMPs) in saliva and gingival tissues of human immunodeficiency virus-seropositive (HIV+) patients are unclear.

METHODS: Enzyme-linked immunosorbent assay methods and Western blots were used to study levels and molecular forms of MMP-7, -8, -9, -25, and -26 and TIMP-1 from salivary samples of HIV+ patients ($n = 55$) and healthy controls ($n = 10$). The expression of MMPs was also studied by immunohistochemical means in gingival tissue specimens ($n = 11$, HIV+ patients; $n = 10$, healthy controls).

RESULTS: The HIV+ patients' MMP-8 levels in saliva were statistically significantly higher only in the acquired immunodeficiency syndrome (AIDS)-phase. MMP-9 levels in ASX- and AIDS-phases showed increased expression. TIMP-1 levels were significantly decreased in lymphadenopathy syndrome (LAS)- and AIDS-related complex (ARC)-phases, while MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were increased in all phases in comparison with controls. The molecular forms of MMP-7, -25, and -26 were different between patients and controls as assessed by Western blot. Immunohistochemical studies showed slightly enhanced MMP-7, -8, -9, -25, and -26 staining in HIV+ gingival tissue samples in comparison with controls.

CONCLUSIONS: This study confirmed and further demonstrated differences in salivary amounts and molecular forms of MMPs and TIMP-1 in HIV+ patients. The results may reflect alterations in host defence reactions associated with HIV infection.

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Keywords: cell markers; enzyme-linked immunosorbent assay; gingiva; HIV; immunohistochemistry; matrix metalloproteinases; Western immunoblot

Introduction

Human immunodeficiency virus (HIV)-1 infection reduces the number of CD4+ T helper lymphocytes in peripheral blood. HIV-1 infection also increases the expression of lymphocyte and monocyte adhesion molecules and T-cell and monocyte matrix metalloproteinase (MMP) secretion associated with the ability of these cells to traverse artificial basement membrane (BM) barriers *in vitro* (1, 2). HIV-1 infection thus enhances the ability of infected lymphocytes to extravasate blood vessel walls and localize in tissues (2). However, there is no clinical evidence that HIV-1-infected individuals would secrete more MMPs (2), except that HIV-1-infected monocytes have been reported to secrete increased levels of MMP-9 (1).

Periodontal inflammatory lesions are characterized by dense infiltrations of immune cells such as activated T- and B-lymphocytes, plasma cells, monocyte/macrophages and mast cells (3, 4). T-cells and plasma cells are also present in the gingival tissue of HIV+ patients (5, 6) and increased numbers of mast cells have been found in HIV+ periodontitis patients (7). Mast cells can express various MMPs (8).

The MMPs are a family of 23 genetically distinct but structurally related Zn^{+}-dependent enzymes. They can be divided into five subgroups according to structural and functional properties. MMPs play a key role in

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normal physiological conditions such as tissue development, morphogenesis, wound healing and apoptosis. MMPs are also important in many pathological processes as a part of the non-immunological reactions that lead to extracellular matrix (ECM) and BM degradation, e.g. arthritis, cancer invasion, breakdown of the blood-brain barrier, tissue ulceration and periodontal disease (9–11). In addition, MMPs can process various growth factors, cytokines, chemokines, cell-adhesion molecules, apoptosis factors and complement components, and thereby regulate cellular communication and immune responses (11, 12). Furthermore, certain MMPs such as MMP-8 and -9 may have unexpected protective, anti-inflammatory characteristics, possibly by regulating inflammatory cell apoptosis, eosinophil recruitment and Th2 responses (13, 14).

In our earlier studies on MMP-1, -2, -3, -8 and -9 increased immunoreactivities were found in saliva of HIV+ patients when compared with healthy seronegative control subjects (15, 16). The present study was conducted to re-evaluate the levels of MMP-8 and -9 and tissue inhibitor of matrix metalloproteinases (TIMP-1) in salivary samples of HIV+ patients and controls by enzyme-linked immunosorbent assay (ELISA), which technique was not available previously for MMPs (15, 16) and to study the levels and molecular forms of MMP-7, -25 and -26 by Western blotting. Furthermore, we investigated by immunohistochemistry the possible differences in MMP-7, -8, -9, -25 and -26 immunoreactivities in gingival specimens between patients and controls. Cell markers for the different cell types were used. Our study hypothesis was that there are differences in the salivary levels and gingival tissue expression of MMPs and TIMP-1 between patients and controls.

Materials and methods

Patients and controls

Clinically healthy gingival tissue specimens were taken from 11 HIV+ patients (mean age with SD 33.8 ± 7.8 years; nine males, two females) during routine wisdom tooth extractions. During the time of biopsy, clinical classification of HIV-infection (according to the WHO classification) was asymptomatic (ASX)-phase, lymphadenopathy (LAS)-phase in nine patients, acquired immunodeficiency syndrome (AIDS)-related complex (ARC)-phase in one patient and AIDS in one patient. The preceding and also actual antiretroviral therapy was mainly with nucleoside analogues, none had highly active antiretroviral therapy (HAART). Of the patients in the LAS-phase six had no antiretroviral therapy, one patient had a combination of zidovudine (AZT) and zalcitabine (ddC) for 2 months before biopsy, one patient had AZT for 3 years initially, then ddC for 1 year and, finally for 2 months a combination of lamivudine (3TC) and saquinavir (SQV). One patient had a combination of AZT and 3TC for 2 years and 1 month. The patient in the ARC-phase had initially monotherapy with AZT for 3 years and 7 months, then for 7 months combined with ddC and for the last 2 months a combination of AZT and SQV. The patient in the AIDS-phase had initially a combination of AZT and ddC for 2 1/2 years, then monotherapy with stavudine (d4T) for 14 months and for the last 2 months a combination of d4T and 3TC.

Clinically healthy tissue specimens were taken from 10 control patients during wisdom tooth extractions (mean age with SD 21.3 ± 4.8, two males, eight females). None of the control patients were taking any medication at the time of the biopsies. All specimens were immediately placed in neutral buffered 10% formalin.

Paraffin-wax stimulated saliva samples [HIV+ patients ($n = 55$) and healthy controls ($n = 10$)] were taken using routine methods of our clinic. For further details and characteristics of the patients, see Mellanen et al. (15).

The study protocol was carried out according to the Declaration of Helsinki and with the approval of the Ethical Committees of the Institute of Dentistry, University of Helsinki and the Helsinki City Health Department. Informed consent was received from each patient before entry into the study.

Single immunohistochemical staining

Immunostainings for MMP-8, -9, -25 and -26 were performed as described by Tervahartiala et al. (17), utilizing the Vectastain® *Elite* ABC KIT rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA). For MMP-7 Vectastain® *Elite* was used. Specific rabbit polyclonal antiserum against human MMP-8 was used as described by Sorsa et al. (18) and Hanemaaijer et al. (19) and against MMP-9 as described by Westerlund et al. (20) and Sorsa et al. (21). The specific monoclonal antiserum against MMP-7 was purchased from Calbiochem (Cambridge, MA, USA) and specific polyclonal antiserum against MMP-25 from Sigma-Aldrich, Inc. (St Louis, MO, USA). Specific antiserum against MMP-26 was kindly provided by Prof. Carlos Lopez-Otin, Department of Molecular Biology and Biochemistry, University Ouiedo, Ouiedo, Spain (22).

Negative control sections were processed using a protocol identical to that described above but by replacing the primary antibody with rabbit IgG [1:800 (Vector Laboratories) with 2% normal goat serum] or 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Negative controls from representative gingival sections were also processed using the following primary/secondary antibody pairs: MMP-8/mouse IgG, rabbit IgG/CD68, rabbit IgG/mouse IgG.

Double immunohistochemical staining

Double-stainings were performed on the MMP-8 and -9 positive single-staining sections. Double-staining was performed after single-staining using Vectastain® *Elite* ABC KIT Mouse IgG (Vector Laboratories). The process continues immediately after single-staining. After incubation with 3-amino-9-ethylcarbazole (AEC), sections were washed and incubated for 30 min with normal horse serum (1:50 in 2% BSA/PBS; pH 7.4). Thereafter, the normal horse serum was washed off,

replaced, and then incubated with CD43 monoclonal mouse anti-human antibody (Dako A/S, Glostrup, Denmark), CD45 monoclonal mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or CD68/macrophage Ab-3 (Clone KP1) monoclonal mouse anti-human antibody (NeoMarkers, Fremont, CA, USA). After washing three times for 5 min with Triton X-100 in phosphate buffered saline (PBST) the sections were incubated with anti-mouse IgG (1:200; Vector Laboratories). After washing and incubating with the avidin–biotin–peroxidase complex (in PBS; Vector Laboratories) for 30 min at 37°C in a humid chamber, the sections were washed and counterstained with Vector SG color. The sections were briefly rinsed with tap water, dipped for 1 s in Mayer haematoxylin, rinsed and mounted in a manner similar to that carried out for single immunohistochemical staining. Negative control sections were processed using an identical protocol but the primary antibody was replaced by mouse IgG (Vector Laboratories). In stainings MMPs are marked by red and inflammatory subgroups by grey.

Enzyme-linked immunosorbent assay
Salivary samples and salivary protein determinations were collected and preformed from HIV+ patients and the healthy controls described (15, 16). Levels of MMPs and TIMP-1 in saliva samples were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits as recommended by the manufacturers. The ELISA kits for MMP-8 and -9 were Quatikine® and for TIMP-1 the DuoSet® ELISA Development System (R & D Systems Inc., Minneapolis, MN, USA). Molar ratios of MMP-8/TIMP-1 and MMP-9/TIMP-1 were calculated from ELISA-data.

Western blot analysis
The analyses of the presence and molecular forms of MMP-7, -25 and -26 were modified from those described by Mellanen et al. (15, 16) and Kivelä-Rajamäki et al. (23). ECL® Western blotting kits were used as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The specific monoclonal antiserum for MMP-7 (17, 23) and polyclonal antisera for MMP-25 and -26 (22) were the similar to those described in *Single immunohistochemical staining*. The antibodies against MMP-25 and -26 have been recently characterized (22, 24, 25). For MMP-8 and -9, secondary antibodies were conjugated with alkaline phosphatase and for MMP-7, -25 and -26, horseradish peroxidase was used.

The relative amounts of molecular forms of the MMPs were analysed by densitometric scanning with a Model GS-700 Imaging densitometer using the Molecular Analyst program (Quantity One; Bio-Rad Laboratories, Hercules, CA, USA). Data are expressed in densitometric units (DU) (26).

Semiquantitative analysis of immunohistochemistry
The immunoreactivities of MMP-7, -8, -9, -25 and -26 and cell markers CD43, -45 and -68 were semi-quantified into four categories (0, none; +, mild; ++, moderate; +++, abundant) (27).

Statistics
Quantitative differences between patients in the different HIV phases and controls were tested by two-way analyses using Student's *t*-tests. Quantitative differences between molar ratios were tested with Levene's tests for equality of differences.

Results

ELISA analyses for salivary MMP-8 and -9 and TIMP-1
The MMP-8 levels in saliva were statistically significantly higher in patients than in controls only in the AIDS-phase ($P < 0.05$; Fig. 1a) while the mean MMP-9 showed no statistically significant differences between patients and controls (Fig. 1b). TIMP-1 amounts were statistically significantly lower than in controls in all HIV-phases except the ASX- and AIDS-phases (Fig. 1c). MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were increased in all phases of HIV infection in comparison with controls (Fig. 2).

Western immunoblot analysis of MMP-7, -25 and -26
Saliva samples of ARC- and AIDS-phases contained all molecular forms of MMP-7 immunoreactivities:

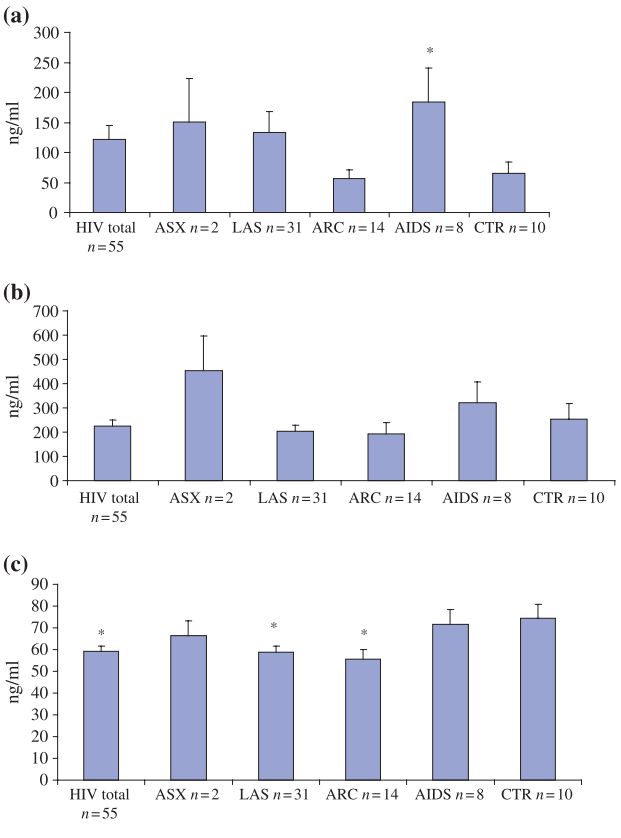

Figure 1 MMP-8 (a) and -9 (b) and tissue inhibitor of matrix metalloproteinase (TIMP-1) (c) levels in saliva from different phases (ASX, LAS, ARC and AIDS) of HIV-infected patients and HIV seronegative healthy controls (CTR). Values were measured with ELISA-technique and expressed as means ± SEM. $*P < 0.05$; $**P < 0.005$; $***P < 0.0001$.

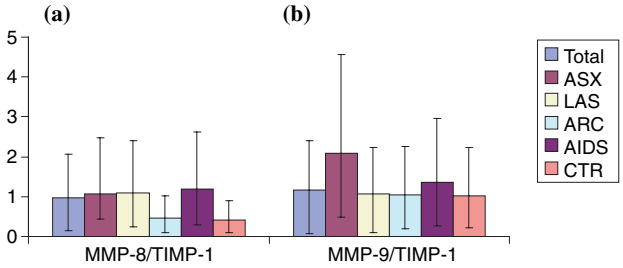

Figure 2 Salivary molar ratios of MMP-8[M]/TIMP-1[M] (a) and MMP-9[M]/TIMP-1[M] (b) calculated from molarities from different phases of HIV infection (ASX, LAS, ARC and AIDS) and from healthy controls (CTR). Values are expressed as means ± SEM.

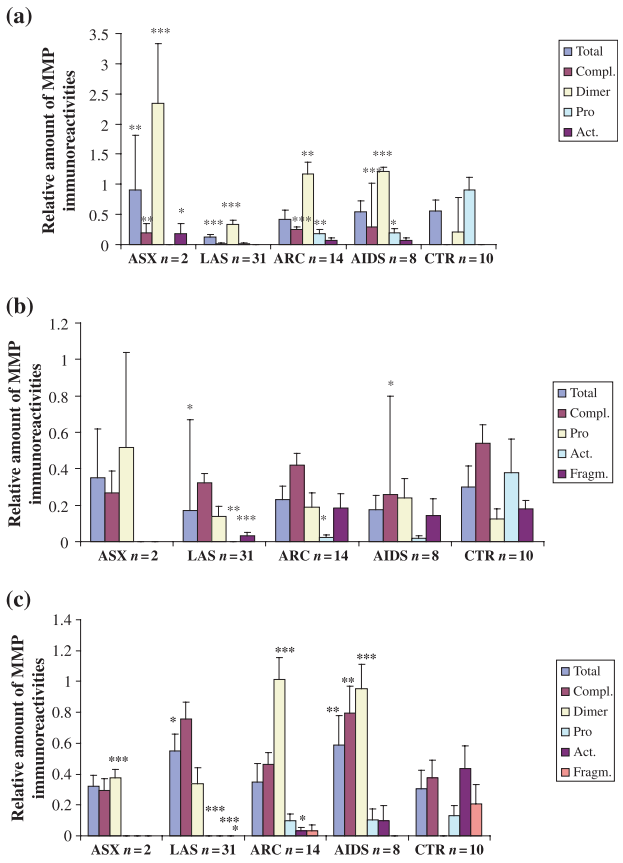

Figure 3 Immunoreactivities for MMP-7 (a), -25 (b), and -26 (c) in salivary samples from different phases (ASX, LAS, ARC and AIDS) of HIV infection and of HIV seronegative healthy subjects (CTR). Values (densitometric units) are means ± SEM. $*P < 0.05$; $**P < 0.005$; $***P < 0.0001$.

complex (>60 kDa), dimeric (40–50 kDa), pro (30 kDa) and active (19–20 kDa) forms (Figs 3a and 4a). The ASX-phase lacked the pro-forms and the LAS-phase lacked the active-forms. No complex or active forms were detected in the salivary samples of controls. The differences between the relative amounts of immunoreactivities of MMP-7 are detailed in Fig. 3a.

Control subjects and ARC- and AIDS-phase patients all had MMP-25 molecular forms in their salivary samples: complex (>60 kDa), pro (50 kDa), active

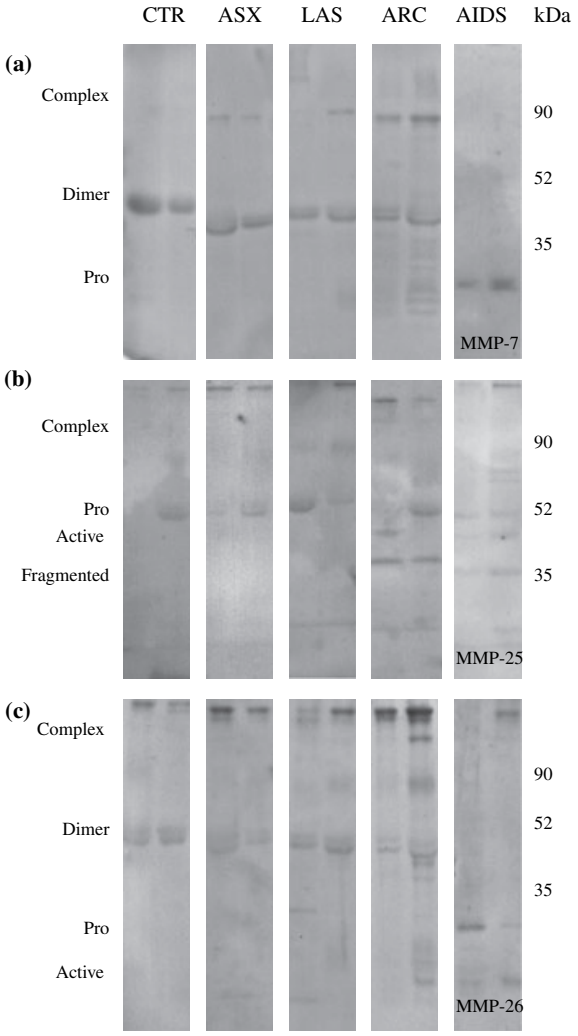

Figure 4 Representative Western blots of immunoreactivities for MMP-7 (a), MMP-25 (b) and MMP-26 (c) in salivary samples from patients in different phases of HIV infection (ASX, LAS, ARC and AIDS) and from control subjects (CTR). The molecular weights (kDa) of standards are indicated on the left.

(40 kDa) forms and their fragments (25–35 kDa). ASX- and LAS-phase patients had low levels or lacked the active forms and fragments, respectively. Moreover, in the ARC- and AIDS-phases the patients had low levels of active-forms. All HIV+ patient samples showed pro-form immunoreactivities but there were no differences when compared with the controls. The details are given in Figs 3b and 4b.

Only ARC-phase patients had all molecular forms of MMP-26 (Figs 3c and 4c). Total amounts of MMP-26 were higher in all HIV-phases, but statistically significantly higher only in the LAS- and AIDS-phases as shown in Fig. 3c. In controls the molecular forms were mostly of the 19–20 kDa active-forms, although statistically significantly higher only when compared with the LAS-phase. In the patients the molecular forms were mostly of the 40–50 kDa dimeric species, which were not seen in controls. These were statistically higher in all the HIV-phases except in the LAS-phase. Complex forms

were also increased in the HIV+ patients except in the ASX-phase (Figs 3c and 4c).

Immunohistochemical findings of MMPs in gingival tissue
In gingival tissue sections there was a strong detectable staining reaction of MMP-8 in neutrophilic leucocytes in connective tissue in three of 11 (27%) HIV+ patients and particularly distinct staining in two of 10 (20%) of the controls. MMP-8-positivity in neutrophils were also found infiltrated both in the oral and sulcular epithelium, in subepithelial BM and also in gingival fibroblasts in the connective tissue (not shown).

In six of 11 (54.5%) of the HIV+ patients and two of 10 (20%) of the controls positive immunoreactivity in tissue sections for MMP-9 was detected. MMP-9-stained PMN leucocytes were found in the subepithelial connective tissue of both the patients and controls and also infiltrating the oral- and sulcular epithelium. HIV+ patients also had MMP-9 immunoreactivity in the perivascular area (data not shown).

Staining for MMP-7 was found in nine of 11 (82%) of the HIV+ samples and in all of the control samples. MMP-7 staining was seen strongly in endothelial cells, in keratinocytes, in fibroblasts and in cells of the lymphocyte lineage (i.e. plasma cells, lymphocytes and macrophages; Fig. 5a–e).

Staining for MMP-25 was found in 10 of 11 (91%) of the HIV+ samples and in nine of 10 (90%) of the control samples. In the HIV+ patients staining was stronger in cells of the lymphocyte lineage and weaker in the endothelial cells than in the controls. MMP-25 was found in BM of HIV+ samples and in a few fibroblasts in both the groups (Fig. 6a–f).

Staining for MMP-26 was seen in all HIV+ and control samples. In all samples of both groups strong reactions were seen in the collagen fibres of connective tissue. In the HIV+ samples MMP-26 was seen in the proliferating part of the oral epithelium (basal cell layer and suprabasal keratinocytes) and in the endothelial cells (Fig. 6g–i).

Double staining with CD43, -45 and -68
All three cell markers were detected in the connective tissue of HIV+ patients and controls. CD45 was detected in lower density. CD43 and CD68 markers were both detected in the oral and sulcular epithelium of HIV+ samples, but in controls they were detected only in the oral epithelium. CD43 and CD68 were occasionally detected in the same cells, which also showed positive immunoreactivity for MMP-8. CD45 was detected without MMP-8 detection. CD68 and CD43 were occasionally detected in the same cells that had MMP-9 immunoreactivity but only in the HIV+ samples. CD45 detection was seen without simultaneous detection of MMP-9 (Fig. 7a–f).

Discussion

The results of the present study showed that the expression and amounts of MMPs and TIMPs in saliva and gingival tissue samples varied in patients at different phases of HIV infection and that there were differences between the patients and controls, as expected. To our knowledge, this was the first comprehensive investigation analysing a number of different MMPs in oral samples of HIV-infected patients. However, the weakness of our study was the small number of patients at the different phases of infection. For more patient material a multi-centric approach might be needed.

We consider that the findings concerning both saliva and gingival tissue samples of the present study without HAART therapy present a native situation in HIV infection. Non-HAART therapy, according to the knowledge of today, did not have any or had only marginal effect on the HIV-load and on the overall clinical situation.

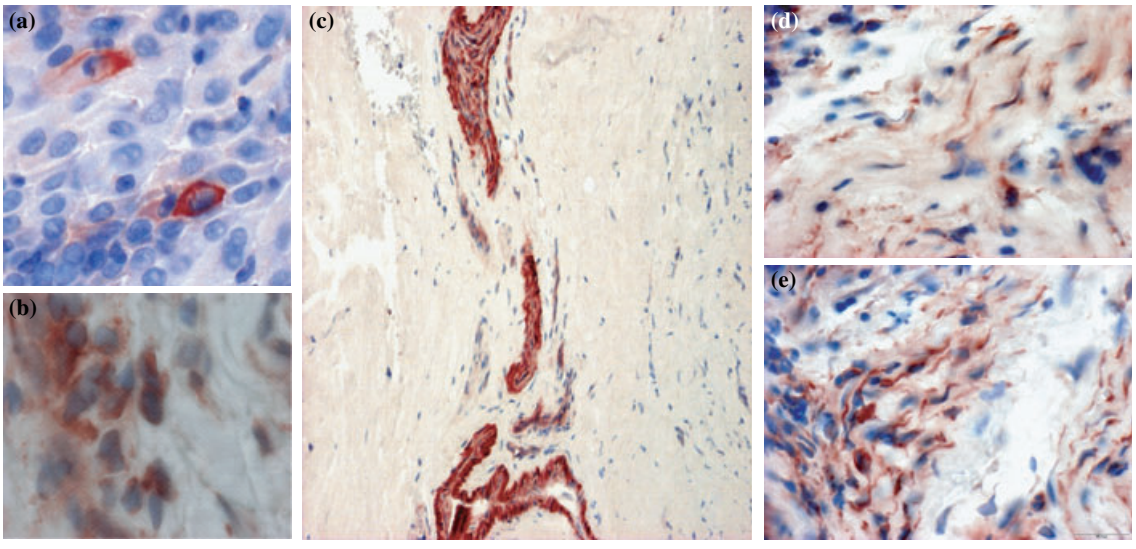

Figure 5 Immunolocalization of MMP-7 in gingival specimens of HIV+ patients and control subjects. MMP-7 immunolocalization in keratinocytes (a) and monocyte/macrophages in HIV+ patients' gingiva (b). MMP-7 immunolocalization in endothelial cells in the gingiva of control subjects (c). Overview of HIV+ patient (d) and control (e) connective tissue with MMP-7 immunolocalization in gingival fibroblasts.

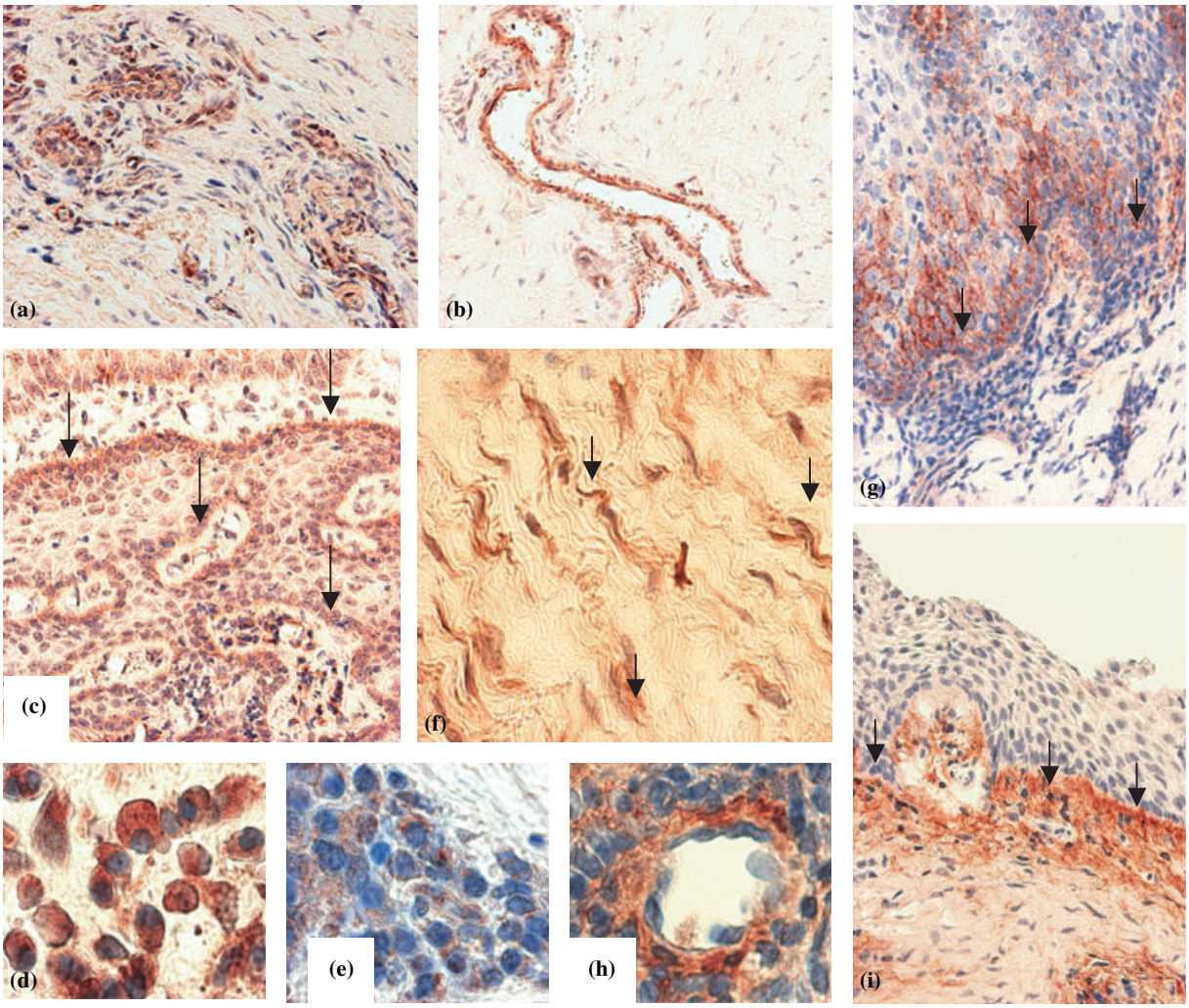

Figure 6 Immunolocalization of MMP-25 and -26 in the gingival specimens of HIV+ patients and control subjects. MMP-25 immunoreactivity near endothelial cells in HIV+ patients' (a) and controls gingiva (b). MMP-25 immunoreactivity in the basal cell layer of an HIV+ patient (arrowheads) (c). MMP-25 immunoreactivity in leukocytes in connective tissue of HIV+ patients and controls (d, e). MMP-25 immunoreactivity in control connective tissue (arrowheads) (f). MMP-26 immunolocalization in connective tissue underneath oral epithelium in HIV+ patients' gingival tissue (arrowheads) (g). MMP-26 immunolocalization close to endothelial cells in control subject tissue (h). MMP-26 immunolocalization in basal cell layer (arrowheads) in HIV+ patients' gingival tissue (i).

Immunological defects caused by HIV infection have been proposed to be responsible for severe forms of periodontal disease pathologies such as necrotizing ulcerative gingivitis (28). On the other hand, the frequency of HIV-associated periodontal disease seems to be lower today than suggested earlier (29). Severely immunocompromised (CD4+ lymphocyte counts <200 cells/μl) HIV+ patients have shown less severe periodontal inflammation than expected (30), although HIV infection is associated with an increased number of mast cells, macrophages and neutrophils in chronic periodontal lesions (6, 7). Nevertheless, HIV+ patients with low CD4+ lymphocyte counts are more likely to develop oral manifestations such as destructive periodontal infections (31). Based on *in vitro* studies, it has been hypothesized that HIV-1 can increase the number of lymphocytes and monocytes in tissues leading to release of HIV-1 proteins causing local tissue damage (2). Furthermore, HIV infection has been shown to increase MMP expression in leukocytes and to further stimulate T-cell and monocyte invasiveness in cell cultures (2).

In our 2-year follow-up study of HIV+ patients we found that the patients had a tendency to develop periodontitis (32). This is in agreement with earlier studies on the activation and levels of salivary MMPs and myeloperoxidase (15, 16). In these earlier studies Western immuno- and dot-blotting were used to assess salivary MMPs, because no MMP- or TIMP-ELISA methods were commercially available at the time of investigation (15, 16). ELISA methods are still not available for all the family members of MMPs and, subsequently, it is not possible to analyse the different molecular forms and the degree of their activation using ELISA, which can detect or target solely the total immunoreactivity of the studied MMPs or TIMPs. Therefore, in the present study, we analysed by ELISA the MMP-8, -9 and TIMP-1 levels and used Western

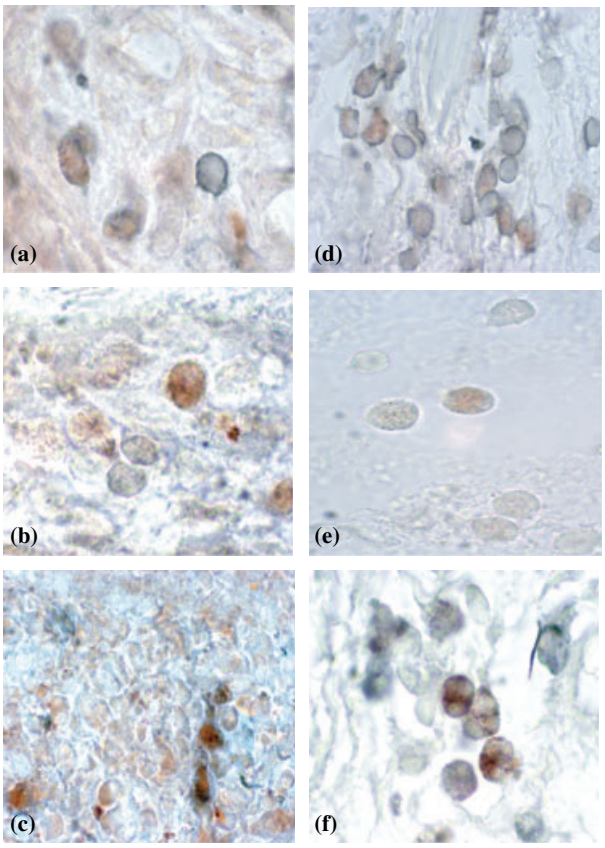

Figure 7 Double staining with MMP-8 and CD43 (normal/neoplastic T cells and mast cells) (a), double staining with MMP-8 and CD45 (B and T cells) (b), and double staining with MMP-8 and CD68 (macrophages) (c). Double staining with MMP-9 and CD43 (normal/neoplastic T cells and mast cells) (d), double staining with MMP-9 and CD45 (B and T cells) (e) and double staining with MMP-9 and CD68 (macrophages) (f). MMP and CD-positive cells are indicated with arrows. MMPs are marked by red and inflammatory subgroups by grey.

immunoblotting for analysing the levels, molecular forms and degree of activation of MMP-7, -25 and -26. By immunohistochemistry, we further analysed the expression of these MMPs in gingival tissue specimens.

MMPs were previously considered to be the pivotal mediators of tissue destruction (11, 15, 16). However, recently, MMPs and especially MMP-7, -8 and -9 have been shown to exert unexpected anti-inflammatory or protective characteristics eventually because of their capacity to process anti-inflammatory cytokines and chemokines (13, 33). MMP-7 has further been shown to convert prodefensins to active defensins, which participate in the protection of mucosal membranes (34, 35). MMP-8 has been shown to be an anti-inflammatory factor, especially in LPS- and allergen-induced lung inflammation (13, 14). The relevance of these findings in periodontal disease of the HIV-infected patients is not known, however.

Our previous studies on salivary MMP-8 and -9 levels of HIV+ patients and healthy controls demonstrated that both these MMPs were slightly elevated and converted to active forms (15, 16). However, the present ELISA assessment showed that only in the AIDS-phase the increase in MMP-8 level was statistically significant. The slight differences regarding the total salivary levels of MMP-8 and -9 in previous studies using Western blot analysis (15, 16) and the present ELISA recording could be explained by the apparent differences in sensitivities of the techniques and/or antibodies used (15, 16). Salivary TIMP-1 levels in LAS- and ARC-phase patients were statistically significantly lower while the salivary molar ratios of MMP-8/TIMP-1 and MMP-9/TIMP-1 were higher in all HIV-phases. These findings suggest that in all subjects the increased levels of both MMP-8 and -9 were converted to active forms and that activated MMP-8 and -9 could possibly overcome the endogenous protective anti-proteolytic shield by the partially fragmented and probably inactivated TIMP-1. Thus the apparent molecular imbalance detected between MMP-8 and -9, the major MMPs associated with the progression of periodontal disease (11) and their major endogenous inhibitor TIMP-1 may explain, or reflect, the liability of these patients to periodontal disease. On the other hand, these findings may also reflect, at least in part, the anti-inflammatory or defensive processes against HIV infection in saliva (13, 14).

The differences in MMP-8 and -9 immunostaining of the gingival tissue samples were not as large as one would expect between patients and controls. Nevertheless, more infiltrating PMN cells in gingival connective tissue and in oral and sulcular epithelium samples stained for MMP-8 and -9 in the HIV+ tissue than in controls. Additionally, more connective tissue fibroblasts of the HIV+ patients stained for MMP-8 and -9. Increased number of plasma cells and inflammatory cells have been found in HIV+ patients with chronic or marginal periodontitis, respectively (6, 7).

Cell markers for CD43 and CD68 showed staining most frequently in the HIV+ samples while double staining revealed expression of MMP-8 and -9 in normal/neoplastic T cells and mast cells (CD43) and in macrophages (CD68). Our findings differ from those of Myint et al. (6, 7) and Næsse et al. (4) who observed more mast cells and enhanced MMP expression in HIV+ patients with clinically healthy gingiva.

In the present study we measured MMP-7, -25 and -26 by Western immunoblotting because of the small salivary sample volume. MMP-7, unlike other MMPs, is expressed by non-injured, non-inflamed exocrine and mucosal epithelium similar to that in salivary glands. MMP-7 (matrilysin) can be induced by bacterial exposure (34, 35). Increased immunoreactivities of MMP-7 mainly in the 19–21-kDa active and 28–30-kDa pro-MMP-7 forms (23) have also been found in peri-implantitis and peri-mucositis sulcular fluid reflecting the stage and course of peri-implantitis and in the gingival crevicular fluid (GCF) of untreated periodontitis patients (17).

Western blot analyses showed that there were no differences in the total amounts of MMP-7 in the salivary samples of patients and controls. This result may not reflect periodontal or other bacterial infection

but rather constitutive MMP-7 expression in the non-inflamed oral tissues (34). However, HIV infection has been found to enhance MMP-7 expression, activation and levels in the cerebrospinal fluid together with MMP-2 and -9. MMP-7 has also been related to HIV-associated dementia (36).

In our patients MMP-7 was present mostly in the dimeric and complexed form, reflecting the *in vivo* activation of the enzyme in the extracellular milieu (37). Immunostainings of gingival tissue samples hardly revealed any differences in the levels of MMP-7 between patients and controls. MMP-7 immunoreactivity was seen in endothelial cells, fibroblasts, cells in the lymphocyte lineage and in the proliferating epithelium. The strongest MMP-7 immunoreactivity was seen in endothelial cells. In ulcerative colitis MMP-7 expression has been correlated to the degree of inflammation (38), but in a study of periodontitis patients MMP-7 was only slightly expressed in the gingival tissue when compared with healthy controls (17). MMP-7 can also activate proMMP-8 and -9 as well as anti-microbial prodefensins (39) that participate in periodontal tissue destruction (11) and/or in protective mucosal defence cascades (13, 14).

The MMP-25 (leukolysin) or MT6-MMP is stored in a latent form in intracellular granules of peripheral blood leukocytes (neutrophils) similar to MMP-8 and MMP-9 (11, 40). It can be rapidly released into the extracellular milieu by stimulation and it promotes tissue remodelling or destruction (40). MMP-25 is also expressed in some brain tumours and is capable of activating proMMP-2 (41). MMP-25 is important both in host defence and in tissue damage (40). In the present study, soluble MMP-25 was detected at higher amounts in its active form in controls while in HIV+ patients MMP-25 was almost non-existent in all phases of infection.

This is the first report describing the soluble or shed forms of MMP-25 in saliva of HIV+ patients and healthy controls. Previously, soluble or shed forms of MMP-14 (MT1-MMP) have been found in periodontitis-related GCF, inflamed human tear fluid, in bronchoalveolar lavage samples from bronchiectasia patients, as well as in breast carcinoma, mesangial cells and in gingival fibroblast culture media (17, 42, 43). MMP-25 can possibly be released into soluble or shed forms similar to MMP-14 (17, 42, 43). The shedding may be associated with enhanced activation involved in periodontal inflammation and autocatalysis (43). MMP-25 is predominantly expressed in leukocytes (40), as observed in the present study. MMP-25 may also function as a proteolytic tool for leukocyte chemotaxis during inflammatory process (44).

The MMP-26 has recently been detected in peripheral leukocytes, lymphocytes, macrophages and in fibroblasts (45, 46). In our study, the total amount of MMP-26 was higher in saliva samples of all HIV patients. It was mostly detected in the dimeric form in patients, but not in controls. However, strong immunostaining in the collagen fibres of connective tissue was found in all gingival tissue specimens of both the study groups. MMP-26 staining was occasionally also seen in the proliferating epithelium and in vascular endothelial cells similar to a recent study on necrotizing enterocolitis (47). Li et al. (48) and Uria and Lopez-Otin (22) have suggested that MMP-26, especially by cleaving α-1-antitrypsin, an inhibitor of neutrophil elastase and activating proMMP-9, can contribute to and promote the coordinated interplay of these two proteinase groups to work in concert and cascades at the sites of inflammation. In this respect MMP-26 might be an inflammatory mediator and a target enzyme in pathological situations (49).

Although the total amounts of MMP-7, -25 and -26 assessed by Western blotting were significantly increased only as regards MMP-7 in the ASX-phase and MMP-26 in the AIDS-phase, the differences in the molecular forms were evident between HIV+ patients and controls. The finding may reflect altered host defence associated with HIV infection.

In conclusion, our ELISA and Western blot analyses together with immunohistochemical findings demonstrated that HIV infection could predispose to periodontal destruction. Alternatively, defensive reactions may, at least in part, be mediated by MMPs also among the HIV-infected persons (13, 14). However, further studies with larger patient materials are needed to clarify the observed differences in the molecular forms and expression of MMPs. Their relevance to the clinical situation also calls for more studies (50).

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