72-kDa and 92-kDa gelatinases in saliva of patients with human inmmunodeficiency virus infection

Liisa Mellanen, Tuula Salo, Tuula Ingman, Yrjö Tapio Konttinen, Juhani Lähdevirta and Timo Sorsa

Department of Periodontology and Department of Pedodontics and Orthodontics, Institute of Dentistry, and Department of Anatomy, Institute of Biomedicine, University of Helsinki; Department of Medicine Division of Rheumatology, Helsinki University Central Hospital; and Aurora Hospital, Helsinki; and Departments of Oral Surgery and Pathology, Institute of Dentistry, University of Oulu, Oulu; Finland

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Human immunodeficiency virus (HIV) infection has been associated with periodontal diseases in HIV-seropositive patients. In periodontal diseases, matrix metalloproteinases (MMPs) may play key roles in the extracellular matrix, basement membrane, serpin degradation, and modification of cytokine action. We characterized the 72 kDa type IV collagenase (gelatinase A, MMP-2) and 92 kDa type IV collagenase (gelatinase B, MMP-9) in the saliva of HIV-seropositive patients and seronegative healthy controls by activity measurements and quantitative immunoblotting. Immunoblot analysis with specific antibodies against MMP-2 and MMP-9 and their tissue inhibitors (TIMP-1, TIMP-2) disclosed that, independent of the phase of the patients' HIV infection, their salivary samples contained higher amounts of MMP-2 and MMP-9 immunoreactivities in pro- and active forms and the TIMP-1 and TIMP-2 inhibitors than did the control samples. Healthy control saliva contained only slight immunoreactivities for gelatinases and TIMPs. However, as judged by the studied clinical and microbiologic indicators, HIV-seropositive patients showed only a slight tendency to develop periodontitis. Overall, an increased amount of gelatinases in saliva may reflect increased host response and defense activities in HIV infection. □ *Matrix metalloproteinases; oral fluids; periodontitis; type IV collagenases*

Liisa Mellanen, Department of Periodontology, Institute of Dentistry, P.O. Box 41(Mannerheimintie 172), FIN-00014 University of Helsinki, Finland

Numerous oral manifestations have been recorded in patients with HIV infection, and claims have been made about their importance in the early diagnosis of HIV and in assessing prognosis of the disease (1). At the EEC-Clearinghouse meeting in Amsterdam in 1990, oral lesions associated with HIV infection were classified into three groups: 1) lesions strongly associated with HIV infection; 2) lesions less commonly associated with HIV infection; and 3) lesions possibly associated with HIV infection. Altogether, these three groups include over 30 oral lesions and diseases known to be associated with HIV infection (2). Unique forms of periodontal disease such as gingivitis and periodontitis are involved in HIV infection, although their frequency, clinical appearance, and stability vary significantly (3). Matrix metalloproteinases (MMPs), 20 homologous enzymes, can degrade almost all extracellular matrix components in several physiologic and pathologic conditions (4). MMPs are in fact considered the key mediators of extracellular matrix, basement membrane, serpin degradation, and cytokine-modification associated with periodontal and other oral diseases (5–13). Among the MMPs, gelatinases/type IV collagenases are divided into two subtypes: 72 kDa gelatinase A (MMP-2) and 92 kDa gelatinase B (MMP-9). The former is expressed by almost all cell types, including fibroblasts (14), macrophages (15), keratinocytes (14), and osteoblasts (16)—except polymorphonuclear leukocytes (PMNs). The latter is mostly expressed by PMNs (17) but also has other sources, such as macrophages (18), keratinocytes (14), and osteoblasts (16).

In general, MMP expression has been found to differ regarding distinct cell types and induction mechanisms and stimuli. Previous studies have indicated that in systemically healthy adults salivary MMPs reflect severity of periodontitis (19, 20). However, severe systemic diseases such as HIV may modify the host response, and this may reflect in saliva. We thus identified and characterized the molecular species of 72 kDa (MMP-2) and 92 kDa (MMP-9) gelatinases present in saliva of HIV-seropositive patients by quantitative immunoblotting and measured their salivary gelatinolytic activities in relation to systemically healthy, age-matched controls. Since gelatinases, like other MMPs, are inhibited by tissue inhibitors of MMPs (TIMP-1 and TIMP-2), we also used quantitative immunoblotting to identify TIMPs in HIV-seropositive patients' salivary samples.

Materials and methods

Reagents

All chemicals used were of the highest commercial

reagent grade available. Unless stated otherwise, they were purchased from Sigma Chemicals (St. Louis, Mo., USA). The following were kindly provided: polyclonal rabbit antiserum to human fibroblast-type 72 kDa MMP-2 and polyclonal rabbit antiserum to human TIMP-2, by Dr. W. G. Stetler-Stevenson, National Institute of Cancer Research, Bethesda, Md., USA; polyclonal rabbit antiserum to human PMN 92 kDa MMP-9, by Dr. L. Kjeldsen, Department of Haematology, Rigshospitalet, Copenhagen, Denmark (21, 22); and polyclonal rabbit antiserum to recombinant human TIMP-1, by Drs. K. M. Bodden and H. Birkedal-Hansen, Department of Oral Biology, School of Dentistry, University of Alabama, Birmingham, Ala., USA.

Patients

Ten female and 46 male HIV-seropositive patients visiting the Aurora Hospital Dental Clinic, Helsinki, Finland, volunteered for the study. Patients were divided into four categories according to the WHO classification of HIV infection: 1) asymptomatic phase (ASX-phase) ($n = 2$: 1 woman, 31 years; 1 man, 41 years); 2) lymphadenopathy-syndrome phase (LAS-phase) ($n = 32$: 7 women, 25 men; mean age, 37 years; range, 23–68 years); 3) AIDS-related complex phase (ARC-phase) ($n = 14$: 1 woman, 13 men; mean age, 38 years; range, 29–60 years); and 4) acquired immune deficiency syndrome phase (AIDS phase) ($n = 8$: 1 woman, 7 men; mean age, 40 years; range, 30–54 years). CD4 cell counts $\times 10^9$/L of these HIV-infected patients, given as mean $\pm$ standard error of the mean, were 0.82 ± 0.13 in the ASX, 0.42 ± 0.01 in the LAS, 0.17 ± 0.01 in the ARC, and 0.04 ± 0.01 in the AIDS group.

One of the patients had diabetes, and 1 had lichen corneus optusum. Twenty-nine patients were receiving various retroviral drugs: 20 were on AZT (3′-azido-2′,3′-dideoxythythymidine), 4 were on ddI (2′,3′-dideoxyinosine), 5 were on ddC (2′,3′-dideoxycytidine), and 1 was receiving treatment for cytomegalovirus retinitis. Fourteen patients were receiving various anti-fungal medication. Ten were receiving pentamidin-inhalations as profylaxis against pneumocystis carinii pneumonia (PCP). Six were receiving psycopharmatics. Ten were receiving various antibiotics (cephalosporins, trimethoprim-sulfamethoxazole, antimycobacterial medication, rifampicin, ethambutol). All medications were taken during the research period, and some patients had one or more medications at the same time. The drugs used for medication of HIV-infected patients would not affect the catalytic activities of MMP-2 or MMP-9. Fourteen patients were receiving no medication.

Age-matched, systemically healthy control subjects ($n = 10$: 5 women, 5 men; mean age, 36 years; range, 27–58 years) receiving dental care at the Helsinki City Dental Clinic, Helsinki, Finland, were HIV-seronegative and had a healthy periodontium and no detectable oral lesions.

Clinical and microbiologic evaluation of HIV patients and control subjects

Clinical documentation of the periodontium of HIV-seropositive patients and healthy controls was carried out by the standard measurements of the clinical indices pocket probing depths (PPDs), radiographic bone loss, visible plaque index (VPI), bleeding on probing (BOP), and retentive calculus (RC). The PPDs were measured with the WHO probe. Radiographic attachment loss was estimated by detection of alveolar bone level changes exceeding 2 mm from the cemento-enamel junction with a transparent ruler with a millimeter scale from panoramic radiographs (23). The microbiologic examinations (potent periodontopathogenic bacteria and candida) of pooled subgingival samples collected from the six deepest (>4 mm) periodontitis pockets/index teeth were carried out at the Oral Diagnostic Microbiological Laboratory, Institute of Dentistry, University of Helsinki (23, 24).

Study protocols were carried out according to the Declaration of Helsinki and with the approval of the ethics committees of the Institute of Dentistry, University of Helsinki, and the Helsinki City Health Department. All patients included in the study gave their informed consent.

Sample collection

About 5 mL of whole stimulated saliva was collected from the 56 HIV-seropositive patients and 10 systemically and periodontally healthy control subjects. The patients first rinsed their mouths thoroughly with water and chewed paraffin. Salivary samples were immediately processed, frozen, and kept at −20°C until assayed (10, 25, 26).

Measurement of gelatinase activity using soluble radioactive type I gelatin as substrate

Gelatinolytic activity in the studied salivary samples were assayed by ^{125}I-labeled 1.5 μmol/L gelatin as a substrate (27, 28). Salivary samples (20 μg protein) with and without 1 mmol/L aminophenyl mercuric acetate (APMA)- and diothiothreitol (DTT)-treatments were incubated with ^{125}I-labeled gelatin for 1 h at 37°C (27). The undegraded gelatin was precipitated with 20% trichloroacetic acid (TCA). The supernatants were determined by a gamma scintillation counter. Purified type IV collagenases/gelatinases (72 kDa MMP-2 and 92 kDa MMP-9) served as controls (29).

Zymography

The salivary samples (20 μg protein) and 100 ng each of purified MMP-2 and MMP-9 were incubated with the sample buffer for 2 h at 20°C and loaded to 8%–10% SDS-PAGE gels containing 1 mg/mL gelatin as substrate. As molecular weight markers, the standard lane was loaded with low-range Prestained SDS-PAGE Standards

Table 1. Gelatinase activities (mean ± standard deviation) from salivary samples (20 μg protein) of AIDS-related complex phase (ARC-phase) patients in comparison with systemically and periodontally healthy controls

Subjects	^{125}I-labeled type I gelatin degraded (counts × 10^{-3})
ARC-phase patients (n = 10) (−1 mmol/L APMA)	23.4 ± 4.7
ARC-phase patients (n = 10) (+1 mmol/L APMA)	21.2 ± 3.6
Controls (n = 10) (−1 mmol/L APMA)	4.2 ± 1.7
Controls (n = 10) (+1 mmol/L APMA)	5.2 ± 2.4

Salivary gelatinase activities expressed as ^{125}I-labeled gelatin degraded. Saliva was treated with 1 mmol/L aminophenyl mercuric acetate (APMA) for 30 min at 37°C. Supernatants and precipitates were counted separately by gamma scintillation counter as described in the text (Materials and methods).

(Bio-Rad, Richmond, Calif., USA). The electrophoresis was run for 3 h at 4°C with a constant current of 15 mA. After the electrophoresis, the gels were washed for 30 min in 50 mmol Tris-HCl, 2.5% Tween 80 and 0.02% (v/v) NaN_3, pH 7.5; then for 30 min with the same buffer supplemented with 1 mmol/L $ZnCl_2$ and 5 mmol/L $CaCl_2$, 0.02% NaN_3, pH 7.5, at 37°C, and then stained with Coomassie Brilliant Blue R250. The gelatinolytic activities were analyzed as clear bands against the blue background.

Immunoblot

Salivary samples (containing 20 μg protein) were characterized by immunoblotting with specific antisera for MMP-2, MMP-9, TIMP-1, and TIMP-2. The samples were treated with Laemmli buffer, pH 6.8, and heated for 5 min at 100°C. The samples were separated in 8%–10% SDS-PAGE cross-linked gels at 200 V for 30 min and electrophoretically transferred to a nitrocellulose membrane at 80 V for 60 min (Bio-Rad). Gelatin (3%) in 10 mmol/L Tris-HCl, pH 8.0, 0.5% Triton X-100, 22 mmol/L NaCl (TBS), was used to block nonspecific binding sites on the nitrocellulose membranes. After washes with TBS (3 × 15 min) the membranes were incubated with anti-MMP-2 antibody (1:1000 dilution in TBS), with anti-MMP-9 antibody (1:1000 in TBS), and with anti-TIMP-1 and TIMP-2 (1:1000 in TBS) for 10 h. After washes with TBS (3 × 15 min) the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemicals), 1:1000 in TBS, for 1 h. After being washed for 15 min with TBS and 15 min with 10 mmol/L Tris-HCl, pH 8, 22 mmol/L NaCl, the

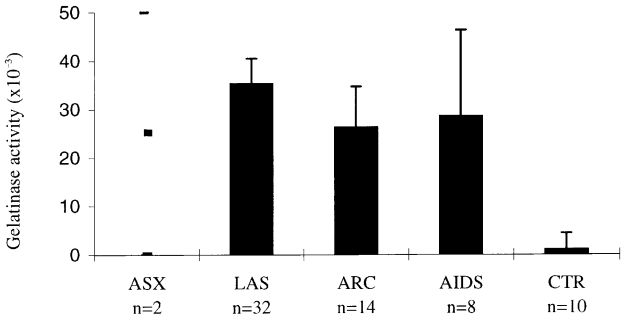

Fig. 1. Gelatinolytic activity in salivary samples of human immunodeficiency virus (HIV)-seropositive patients and healthy controls without aminophenyl mercuric acetate (APMA). Purified 72-kDa gelatinase run as standard. Values are means ± standard error of the mean; for the two asymptomatic (ASX) patients, the actual values and their mean are given. LAS = lymphadenopathy syndrome; ARC = AIDS-related complex; CTR = controls. $P < 0.05$, Student's t test.

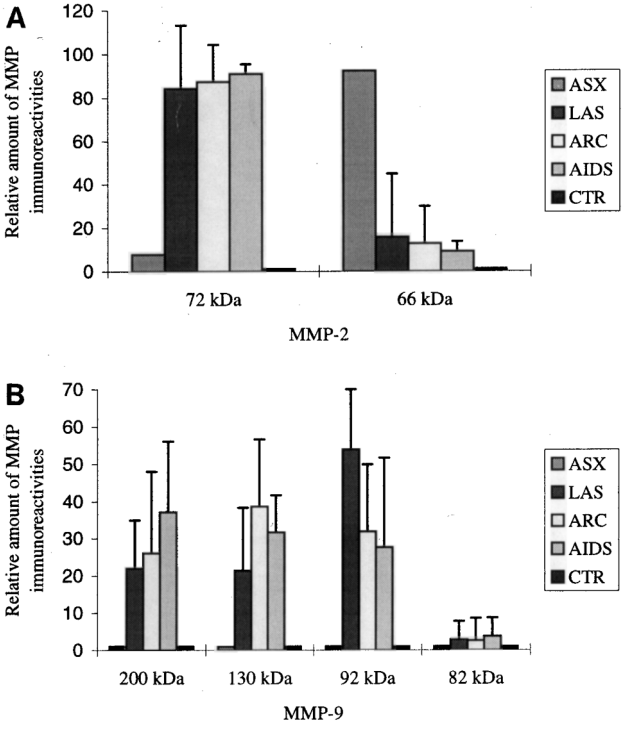

Fig. 2. Higher amounts of molecular species of the matrix metalloproteinases MMP-2 (A) and MMP-9 (B) in saliva of patients in different phases of HIV infection—ASX (n = 2), LAS (n = 32), ARC (n = 14), AIDS (n = 8)—and in that of HIV-seronegative CTR (n = 10) (see Fig. 1 for definitions). Values are means ± standard deviation. Regarding different molecular species of MMP-2 (72-kDa proform and 66-kDa active form) and MMP-9 (polymeric 200-kDa and 130-kDa as well as 92-kDa pro- and 82-kDa active forms), densitometrically detected relative amounts of immunoreactivities in control samples are taken as 1, to which HIV-seropositive groups are compared.

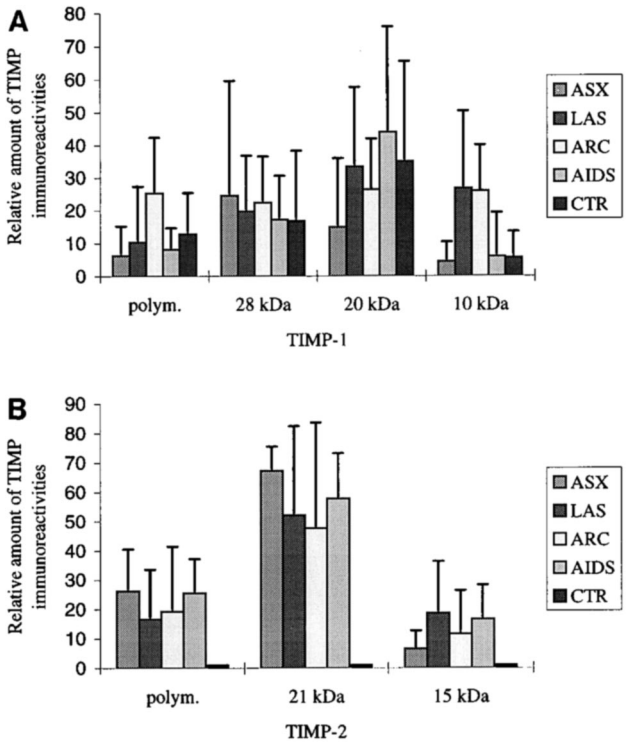

Fig. 3. Immunoreactivities of tissue inhibitors TIMP-1 (A) and TIMP-2 (B) in saliva of patients in different phases of HIV infection—ASX ($n = 2$), LAS ($n = 32$), ARC ($n = 14$), AIDS ($n = 8$)—and in that of HIV-seronegative CTR ($n = 10$) (see Fig. 1 for definitions). Values are means ± standard deviation. TIMP-1 (A) was detected in considerable amounts in the saliva of both HIV-seropositive patients and healthy controls, and the relative distribution of different TIMP-1 species is given. Regarding TIMP-2 (B), a comparison was carried out similar to that for MMP-2 and MMP-9 (see Figs. 2A and 2B). polym. = polymeric.

immunoblots were visualized by addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, diluted to N-N-dimethylformide in 100 mmol/L Tris-HCl, 5 mmol/L $MgCl_2$, 100 mmol/L NaCl, pH 9.5. Molecular weight markers were low-range Prestained SDS-PAGE Standards (Bio-Rad). All incubations were carried out at 20°C. The secondary antibody did not react with the bands detected by immunoblotting.

The relative amounts of latent (pro), active MMP-2 and MMP-9, and different molecular forms (complexed, free, and fragmented) of TIMP-1 and TIMP-2 were obtained by densitometric scanning with the Model GS-700 Imaging Densitometer with the Molecular analyst program (Image analysis system version 1.4, Bio-Rad), taking the intensities measured for controls as 1.0. The relative amounts of MMP-2 and MMP-9 and the TIMP-2 immunoreactivities were related to control values; however, with regard to TIMP-1, only the relative distribution of detected TIMP-1 species is given. Values are expressed as means of relative amounts of MMP/TIMP immunoreactivities.

Statistics

Quantitative differences between groups and controls were tested as a whole by two-tailed analysis of Student's *t* test.

Results

Oral clinical and microbiologic evaluation of HIV patients and healthy controls

VPI, RC, and BOP were significantly higher in the HIV patients than in the HIV-seronegative controls. PPDs, classified as shallow (4–5 mm) and deep (>6 mm) periodontal pockets, were slightly higher in the HIV patients than in the controls. Controls showed some clinical signs of periodontal inflammation/diseases, but they had no deep periodontal pockets, and only a few shallow periodontal pockets were detected.

Putative periodontopathogens—*Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Peptostreptococcus micros*, and *Campylobacter rectus*—were detected in subgingival samples in all of the HIV patients except the eight AIDS patients, six of whom were receiving antibiotics. The principal periodontopathogens, *A. actinomycetemcomitans* and *P. gingivalis*, were not detected in the control samples. The relative amount of candida in periodontal pockets increased with the severity of HIV infection; candida was not found in the healthy controls.

The control patients did not show any radiographic attachment loss. The HIV-seropositive patients demonstrated attachment loss, and there was a statistically significant difference in attachment loss between these groups.

Gelatinase activity

Gelatinase activities were studied in saliva from the HIV-seropositive patients and from the age-matched controls. Salivary samples from HIV-seropositive patients showed significantly higher gelatinase activities, independent of stage of HIV infection, than those of controls (Fig. 1). The optimal organomercurial activator, 1 mmol/L APMA of both MMP-2 and MMP-9, did not activate but rather slightly decreased the salivary gelatinase activities in HIV-seropositive patients; however, slight activation was seen in the control group (Table 1). A similar effect was seen with 1 mmol DTT, a reductant capable of activating proteinases from oral microorganisms (30, 31), with regard to all sample groups studied (data not shown).

Immunoblot analyses of 72 kDa MMP-2, 92 kDa MMP-9, TIMP-1, and TIMP-2 in saliva of patients with HIV infection and in healthy controls

When immunoblot data concerning antibodies for MMP-2, MMP-9, TIMP-1, and TIMP-2 were analyzed, independent of phase of HIV infection, the salivary

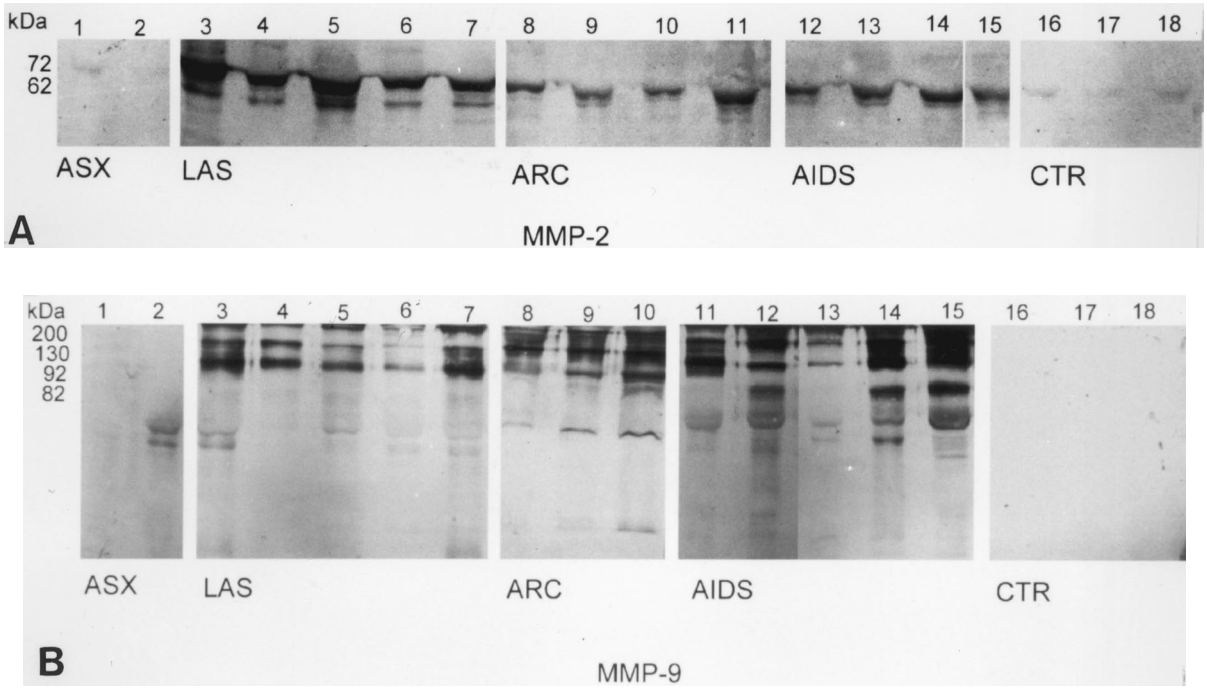

Fig. 4. Immunoreactivities for gelatinase A (MMP-2) and gelatinase B (MMP-9) in salivary samples from patients in different phases (ASX, LAS, ARC, AIDS) of HIV infection and from HIV-seronegative subjects (CTR) (see Fig. 1 for definitions). The molecular weights of standard proteins are shown at left.

samples were found to contain high amounts of immunoreactivities, corresponding to both pro- and active forms of MMP-2 and MMP-9 as well as to TIMP-1 and TIMP-2 (Figs. 2–5). The immunoblot analysis of the control saliva samples showed slight immunoreactivities for proMMP-2 (Fig. 4A), proMMP-9 (Fig. 4B), TIMP-1 (Fig. 5A), and TIMP-2 (Fig. 5B). Densitometric analysis of immunoblots revealed that proMMP-2 was converted to a significant extent to the 66 kDa active MMP-2 species in HIV-seropositive patients' saliva, independent of phase of HIV infection (Fig. 2A). MMP-9 was found to exist mainly in polymeric 200 kDa and 130 kDa as well as in 92 kDa full-size forms (Fig. 2B); but 82 kDa activated and lower molecular weight species were also seen (Figs. 2B, 4B). TIMP-1 and TIMP-2 were present in polymeric, monomeric 28 kDa and 21 kDa, and degraded species (Figs. 3, 5).

Discussion

Midway through the study there were 211 HIV-seropositive patients under care at Aurora Hospital, of which 15 were in ASX-phase (7%), 126 in LAS-phase (60%), 51 in ARC-phase (24%), and 19 in AIDS-phase (9%). This implies that the study population of 56 patients was quite representative of the whole HIV-seropositive patient population at Aurora Hospital. HIV-1 principally infects CD4+ helper T-cells and monocyte/macrophages, but HIV-1 may also infect other host cells (32–34). HIV-1-infection-induced immune dysregulation is associated with marked alterations in cytokine, for example tumor necrosis factor (TNF-α) production (35–38). Thus, HIV-1-infected cells could provide a local reservoir of potentially pathogenic viral gene products, toxins, and cytokines (39). In support of this conjuncture are the increased serum TNF-α levels found in HIV-1-seropositive patients, which evidently reflect the cytokine imbalance of host cells during the course of HIV-1 infection (35–38). HIV-1 infection, which has been shown to up-regulate cytokine/TNF-α-mediated MMP-2 and MMP-9 expressions by human monocytic and mesangial cells (40–43), can further modulate human cells to express TIMPs (40, 41). Both gelatinases (MMP-2 and MMP-9) and TIMPs are involved in extracellular matrix, serpin, and basement membrane degradation and remodeling (4, 39). Such molecular processes are involved in the proteolytic mechanisms associated with invasion and extravasation of host cells (4).

The HIV-infection phases are associated with various oral manifestations, including oral candidosis, hairy leukoplakia, and Kaposi's sarcoma, as well as distinct and severe forms of periodontal diseases (2). Saliva has long been recognized as being potentially valuable material in the detection of oral disease, especially periodontitis (44). Saliva contains a variety of enzymes, including proteases, esterases, lipases, transferases, and glycosidases, from salivary glands, serum leukocytes, oral epithelial cells, and oral bacteria (44). Salivary enzymes participate in the

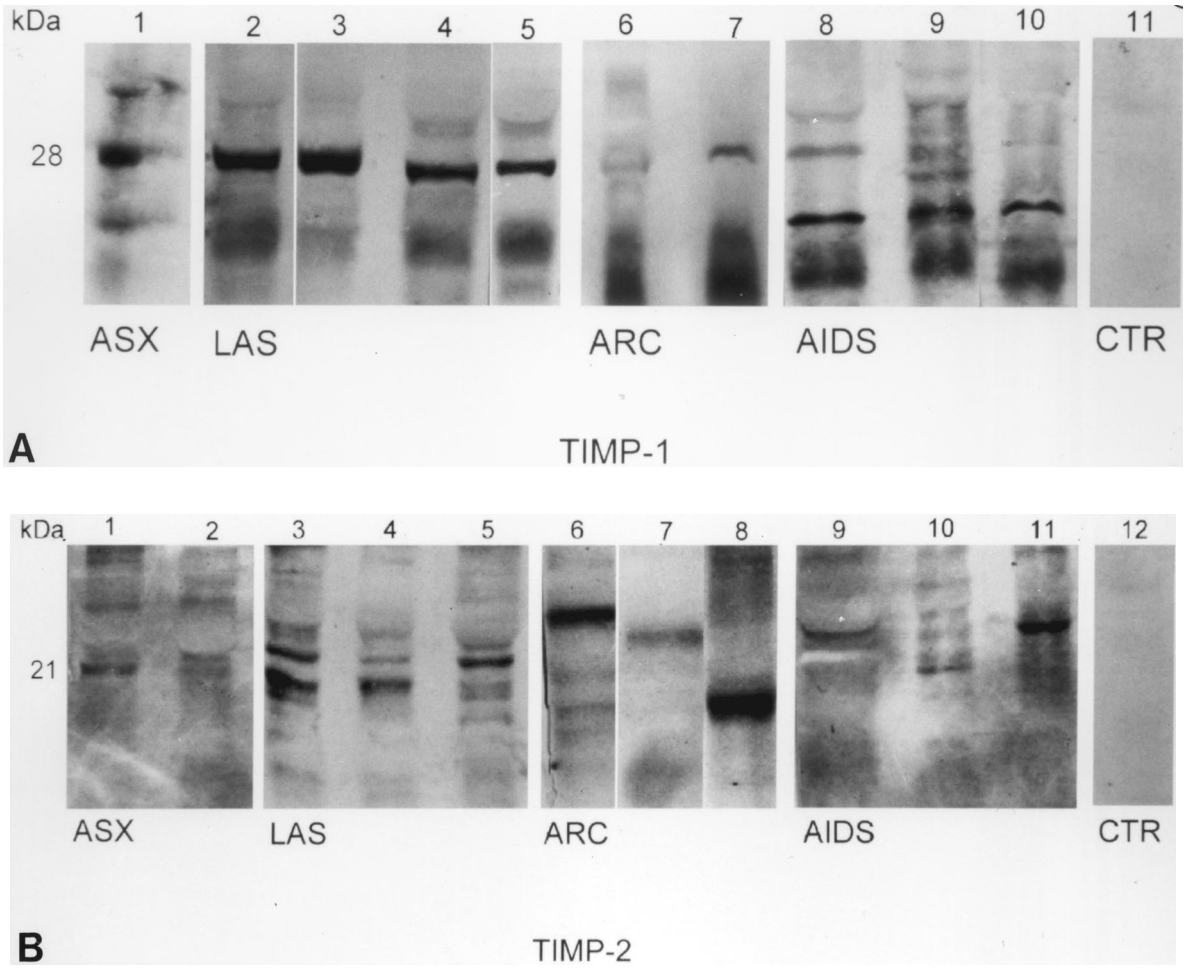

Fig. 5. Immunoreactivities for tissue inhibitors TIMP-1 and TIMP-2 in salivary samples from patients in different phases (ASX, LAS, ARC, AIDS) of HIV infection and from HIV-seronegative subjects (CTR) (see Fig. 1 for definitions). The molecular weights of standard proteins are shown at left.

physiologic processes in the oral cavity, apparently playing a role in the attachment and detachment of oral bacteria and candida (44).

Clinical and microbiologic evaluation demonstrated the presence of putative periodontopathogens such as *A. actinomycetemcomitans* and *P. gingivalis* in the periodontal pockets, with *P. intermedia*, *P. micros*, and *C. rectus* also detected (26). Further, the relative amounts of candida in periodontal pockets increased with the severity of HIV infection (26). The gelatinase activities in HIV-seropositive patients' saliva as assessed by functional assay were in the active form; addition of 1 mmol/L APMA, an optimal organomercurial MMP-activator (12, 29), did not increase but rather slightly decreased the salivary gelatinase activities. APMA is known to partially inactivate endogenously activated MMPs (19, 26, 45, 46). Immunoblot analysis demonstrated that gelatinase A (MMP-2) existed to a significant extent in 66 kDa active/converted species. Immunoblot analysis also demonstrated that MMP-9 existed in polymeric form (200 kDa and 130 kDa), 90 kDa full-size proform, and processed 40–82 kDa form. Regarding proMMP activation, HIV saliva may contain other proteinases capable of activating proMMP-2, but 92 kDa MMP-9 can be activated oxidatively by hypoclorous acid (HOCl) without reduction of the molecular size of the full-length 92 kDa MMP-9 (12). Furthermore, we have recently found that HIV-seropositive patients' saliva, compared with that of healthy controls, contains increased amounts of PMN-derived myeloperoxidase (26) capable of activating proMMP-9 by generating HOCl (12, 47). In the control salivary samples, only slight immunoreactivities representing inactive proforms of MMP-2 and proMMP-9 were detected.

Because salivary protease activities (collagenase, gelatinase, elastase) have been found to correlate with and probably reflect the amount of deep (>6 mm) periodontitis lesions/pockets, salivary enzymes/proteinases might be regarded as useful biochemical markers to screen or monitor periodontal health and status (25). In the present study, the HIV-seropositive patients, irrespective of the

phase of HIV infection, showed high autoactive salivary gelatinase activities and immunoreactivities corresponding to systemically healthy adult periodontitis patients. Nonetheless, most probably owing to the good general and oral health control of the HIV-seropositive patient groups studied, they exhibited very few deep (>6 mm) periodontitis lesions/pockets (26). Even though salivary proteinases have been regarded as reflecting oral and especially periodontal status and inflammation (12, 25), previous studies have found only low amounts of MMP-2 in saliva/GCF/oral fluids/mouthrinse samples of periodontitis patients, and have shown that 92 kDa MMP-9 is the major salivary/oral fluid MMP (4, 6, 10, 12, 20, 29). Regarding HIV infection, however, the present study indicates that salivary proteinases may also to some extent reflect the systemic condition (e.g. a viral infection) that may induce host cells directly and/or via the action of elevated cytokine (TNF-α) levels (35) to express gelatinases, especially MMP-2 and their inhibitor, TIMP-2 (40–43, 48). Alternatively, the increased salivary gelatinase amounts may reflect the Sjögren syndrome–like symptoms, with or without mucosal candida infection, shown to be developed by HIV patients and resulting from mild and subclinical inflammatory changes in the salivary glands (1–3). It is significant that the ability of MMP-2 to attack extracellular matrix-components appears to be greater than previously thought; recent demonstrations show that catalytically competent MMP-2 can degrade native type I collagen in a manner similar to that of classic interstitial collagenases (MMP-1 and MMP-8) (49).

Given our recorded clinical, microbiologic, and biochemical findings, the HIV-seropositive patients, in relation to the HIV-seronegative controls, seemed to be associated with an only moderately increased expression of attachment loss, that is, an irreversible character of destructive periodontal disease (26). Further, the presence, extent, and severity of periodontal diseases among HIV-infected patients may in fact be less than previously generally suggested.

The salivary gelatinase activities and immunoreactivities of HIV-seropositive subjects, regardless of HIV-infection phase, are higher than in periodontally and systemically healthy control subjects. The former group's gelatinase activities were not only higher but in activated form; they did not reflect periodontal status in these patients in a manner similar to that for systemically healthy adult periodontitis patients in previous studies (10, 12, 20, 25, 29). Thus, care should be taken in interpreting results revealing salivary proteinases as diagnostic markers of oral/periodontal inflammation and tissue destruction, because a systemic disease (i.e. HIV infection/AIDS) may also, without any significant involvement of periodontitis, result in increased MMP activities and levels in saliva.

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