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Submandibular Salivary Proteases: Lack of a Role in Anti-HIV Activity

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Abstract. Whole human saliva contains a number of proteolytic enzymes, mostly derived from white blood cells and bacteria in the oral cavity. However, less information is available regarding proteases produced by salivary glands and present in salivary secretions. In the present study, we have analyzed submandibular saliva, collected without contaminating cells, and identified multiple proteolytic activities. These have been characterized in terms of their susceptibility to a series of protease inhibitors. The submandibular saliva proteases were shown to be sensitive to both serine and acidic protease inhibitors. We also used protease inhibitors to determine if salivary proteolytic activity was involved in the inhibition of HIV infectivity seen when the virus is incubated with human saliva. This anti-HIV activity has been reported to occur in whole saliva and in ductal saliva obtained from both the parotid and submandibular glands, with highest levels of activity present in the latter fluid. Protease inhibitors, at concentrations sufficient to block salivary proteolytic activity in an in vitro infectivity assay, did not block the anti-HIV effects of saliva, suggesting that the salivary proteases are not responsible for the inhibition of HIV-1 infectivity.

Key words: saliva, HIV-1, proteases.

Introduction

Since the initial report by Fultz (1986), numerous investigators have confirmed and extended the observation that human saliva contains factor(s) which decrease HIV infectivity (Fox et al., 1988, 1989; Robinovitch et al., 1993; Malamud et al., 1993, 1997; McNeeley et al., 1995; Shine et al., 1997). Anti-HIV activity has been reported in whole, parotid, and submandibular saliva obtained from seronegative individuals, and also in salivary samples from HIV-seropositive individuals. It is of particular interest that while salivary samples are often shown to be “positive” for HIV DNA sequences by PCR techniques (Goto et al., 1991; Liuzzi et al., 1994), infectious virus in these samples is rarely observed (Barr et al., 1992; Moore et al., 1993). These latter observations, combined with epidemiologic data demonstrating a lack of oral HIV transmission, suggest that a salivary anti-HIV factor(s) may block infectivity.

However, the possibility of oral HIV transmission is still in question. For example, Baba et al. (1996) reported that exposure of adult macaques to simian immunodeficiency virus (SIV) by direct application of virus to the tongue resulted in productive infection, raising the possibility of oral transmission in this model. Furthermore, in 12 human subjects where the time of HIV seroconversion could be identified, four (33%) reported that oral-genital contact was their sole risk factor (Schacker et al., 1996).

The nature of the salivary factor(s) inhibiting HIV infectivity remains elusive. Malamud et al. (1993) reported that submandibular saliva could aggregate HIV, suggesting viral entrapment as one possible mechanism. Bergey et al. (1994) demonstrated a potential role for salivary mucins, and Robinovitch reported that anti-HIV activity was associated with basic proline-rich proteins (1993). McNeeley et al. (1995) reported that secretory leukocyte protease inhibitor (SLPI) is present in whole and ductal saliva, and that this protein might block HIV infection by interacting with a cellular protein and thus blocking HIV entry, but this result has been questioned (Turpin et al., 1996). In a study of...
submandibular saliva obtained from 15 seronegative individuals, we found that the anti-HIV activity was quantitatively similar in most of the subjects, a few had low levels of activity, and two individuals showed no detectable anti-HIV activity (Nagashunmugam et al., 1997). Salivary inhibition was specific for HIV-1, with no effects on HIV-2 or SIV. Recent studies suggest that removal of gp120 from HIV exposes gp41, which permits fusion to the cell membrane (Chan et al., 1997). Proteases present in human saliva could potentially cleave gp120 and prematurely trigger the gp41 molecule before it comes into contact with the cell membrane.

In the present study, we have characterized protease activities present in two submandibular saliva samples, one known to inhibit HIV via an apparent agglutination mechanism, and another inhibiting infectivity without viral aggregation. We demonstrate that submandibular saliva does indeed contain multiple proteolytic activities which can be blocked by protease inhibitors effective against either serine or aspartic proteases. However, inhibitor concentrations that block salivary protease activity had no effect on the anti-HIV activity of submandibular saliva. Furthermore, preliminary purification studies indicate that the proteases and the anti-HIV activities reside in different fractions. We conclude that submandibular proteases are not directly involved in anti-HIV activity.

Materials and methods

Saliva

Submandibular/sublingual saliva was collected, by means of a plastic collector custom-fitted for each subject (Stuchell and Mandel, 1978), from two seronegative donors in the morning to minimize diurnal variations. The individuals were selected because one (designated as subject 1) possessed agglutinating anti-HIV activity requiring filtration through a 0.45-μm filter, and subject 2 demonstrated inhibition in the absence of filtration. These two individuals are prototypes for the two types of anti-HIV activity, as previously described (Nagashunmugam et al., 1997). The samples were collected into iced tubes, dialyzed against distilled water, and lyophilized. Prior to usage, aliquots of the lyophilized samples were reconstituted in the appropriate buffers at 2 to 10X concentration relative to the original volume.

Protease assay

For the zymogram assay, 12% polyacrylamide gels containing 0.15% gelatin or casein as substrate were used. Aliquots of lyophilized submandibular saliva were applied to the gels by means of standard SDS-gel loading buffer containing 0.1% SDS, but lacking β-mercaptoethanol, and were not boiled prior to being loaded. Gels were run at 200 volts for 1 hr and soaked in 2% Triton X-100 for displacement of the SDS. Gels were then incubated in reaction buffer (50 mM Tris, pH 8.0, 1 mM CaCl2) for 15 hrs at 37°C and stained in Coomassie blue (Billings and Habres, 1992). Enzyme in the salivary samples digests the gelatin or casein, and after samples are stained, protease activity shows up as clear bands on a blue background.

Total enzyme activity was determined by means of a protease assay kit with resorufin-labeled casein as substrate (Calbiochem). Lyophilized submandibular saliva samples were re-suspended in reaction buffer, pre-incubated (1 hr at 37°C), and assayed for protease activity according to the manufacturers’ protocol (24-hour incubation). Proteolytic digestion of labeled casein was determined spectrophotometrically at 492 nm.

Four classes of protease inhibitors were used to determine the types of protease activity present in saliva. Inhibitors included: the serine protease inhibitors phenylmethylsulfonylfluoride (PMSF), diisopropyl fluorophosphate (DFP) benzamidine-HCl, aprotinin, and Bowman-Birk inhibitor (BBI); the aspartic protease inhibitors pepstatin and diazoacetyl- norleucine methyl ester (DAN); the cysteine (thiol) protease inhibitors leupeptin and N-ethylmaleimide; and the metalloprotease inhibitor, a mixture of ethylenediaminetetra-acetic acid (EDTA)/ethylene glycol tetraacetic acid (EGTA).

The stock solutions of the protease inhibitors were prepared at the following concentrations: PMSF, 100 mM in isopropanol; DAN, 100 mM in cupric acetae; DFP, 1 mg/mL; EDTA, 10 mM plus EGTA 10 mM, and N-ethylmaleimide, 10 mM. The final

Figure 1. Electrophoretic zymogram analysis of submandibular salivary proteases. Gelatin-containing polyacrylamide gels were prepared as described in “Materials and methods”, and two salivary samples, labeled Subject 1 and Subject 2, were analyzed. The salivary samples were used at 10X concentration. Molecular-weight markers are indicated.
concentrations of the protease inhibitors prior to usage were: PMSF, 0.1 mM; DAN and cupric acetate, 5 mM; DIFP, 1 μg/mL; EDTA and EGTA, 10 mM; and N-ethylmaleimide, 0.1 mM. The incubation buffer in the protease assay kit was used as the dilution buffer.

**HIV inhibition**

To determine HIV infectivity, we used HeLa CD4-LTR β-galactosidase cells, as described by Kimpton and Emerman (1992). Cells (8 x 10⁴ per well) were plated in 24-well PVC plates and allowed to attach overnight at 37°C in 1 mL of DMEM, supplemented with 10% fetal calf serum. The media were removed and replaced with: (1) virus + saliva, (2) virus + media, (3) virus + media + protease inhibitor, or (4) virus + saliva + protease inhibitor in the following volumes: 100 μL of HIV-1IIIB (5.6 ng p24), 100 μL of saliva (0.77 mg protein/mL), and 200 μL of protease inhibitors. The dilutions were made in DMEM, and the volume was maintained at 400 μL. The plates were incubated for 3 hrs at 37°C, after which an additional 1 mL of DMEM was added to each well; the plates were re-incubated at 37°C for 48 hrs. The cells were fixed in 2 mL of a 1% formaldehyde/0.2% glutaraldehyde PBS solution, washed twice with PBS, and stained with X-gal. Virus infection of HeLa CD4 results in activation of the LTR and β-galactosidase expression. Positive cells stained blue in the nuclear region and were counted at 100X magnification. The total number of blue cells in each well was counted, and the percent inhibition of infectivity was determined by comparison of saliva-treated virus with virus that was exposed to media only.

**Results**

Analysis of samples by gelatin or casein zymography showed that both saliva donors displayed multiple bands of proteolytic activity (Fig. 1). Saliva from subject 2 had a greater amount of total protease activity and also demonstrated a larger number of lower-molecular-weight forms (<75 kDa).

To determine the responses of salivary proteases to inhibitors, we analyzed submandibular saliva samples obtained from the two individual donors, using resorufin-labeled casein as substrate. In the initial screen, 5 serine protease inhibitors (DIFP, PMSF, Benzamidine-HCl, aprotinin, and BBI), two cysteine protease inhibitors (leupeptin, NEM), two acidic protease inhibitors (DAN, pepstatin), and an EDTA/EGTA mixture as a metalloproteinase inhibitor were evaluated. Both DIFP and PMSF demonstrated strong inhibition, as did pepstatin and DAN, suggesting that the proteases present in the two submandibular saliva samples were serine and acidic proteases.

Selected protease inhibitors were then tested against both saliva samples at a series of concentrations. As shown in Fig. 2, DAN, PMSF, and DIFP each demonstrated the ability to inhibit protease activity, while EDTA/EGTA exhibited only modest and incomplete inhibition. Saliva obtained from...
subject 1 (filtration-dependent) was more sensitive to inhibition with each of the inhibitors compared with saliva from subject 2.

Of the ten protease inhibitors tested, only 4 could be used in anti-viral assays. The other inhibitors were toxic to the cells at concentrations below their effective level as protease inhibitors, and therefore could not be evaluated. The 4 protease inhibitors included one serine protease inhibitor (PMSF), one acid protease inhibitor (DAN), and two compounds which were not effective inhibitors of salivary protease activity (BBI and EDTA/EGTA). We tested each inhibitor in the saliva-HIV assay to determine if they would reverse the effect of saliva on HIV infectivity. As shown in Fig 3, when we used HeLa CD4 cells and monitored the number of β-galactosidase positive cells, over a wide range of concentrations, including levels of PMSF and DAN known to inhibit salivary protease activity, there was no change in the ability of saliva to inhibit HIV infectivity. We confirmed these studies in PBMCs using a single concentration of each protease and measuring p24 antigen levels at 7 days post-infection. Once again, salivary inhibition of HIV infectivity was maintained in the presence of all 4 protease inhibitors (data not shown), demonstrating that anti-HIV activity in these salivary samples is not associated with the protease activity.

To investigate further the relationship between salivary protease activity and salivary anti-HIV activity, we fractionated saliva samples by anion exchange chromatography using HPLC. In these assays, all of the protease activity appears in the void volume of the column, while all of the anti-HIV activity is included in the fractions eluted with increasing salt concentration, suggesting that the proteases are cationic while the HIV inhibitory activity is anionic (data not shown).

**Discussion**

Based on the zymogram and spectrophotometric assays, human submandibular saliva was observed to display multiple proteolytic activities, demonstrated by numerous bands on the zymogram assay, and sensitivity to multiple inhibitors in the spectrophotometric assay. The observation that two classes of protease inhibitors totally inhibit salivary protease activity (Fig. 2) suggests that both serine and acid proteases are present in saliva. The moderate and incomplete inhibition seen with EDTA/EGTA suggests that there may also be metalloproteases in submandibular saliva.

The saliva samples used in these studies produce an 80 to 90% decrease in infectivity when incubated with HIV-1. However, the inclusion of protease inhibitors, at concentrations inhibiting protease activity by 30 to 100%, had no effect on the salivary inhibition of HIV infectivity. The Bowman-Birk inhibitor, which did not block salivary protease activity, was also ineffective in blocking the effects of the salivary anti-HIV factors. None of the protease inhibitors had any effect on HIV infectivity in the absence of saliva (data not shown). These findings suggest that the proteolytic activity of saliva is not directly involved in the inhibition of HIV.

Saliva is known to contain protease inhibitors including...
cystatins and SLPI. It is possible that there is an equilibrium between the activities of proteases and protease inhibitors in the oral cavity. To resolve this issue, we are continuing to examine protease activity in the zymogram assay, where proteases are separated from potential protease inhibitors. In addition, when the anti-HIV activity is purified from submandibular saliva, it will be possible to assess, independently, the proteolytic and protease inhibitory activities of this fraction. It is possible, for example, that there is a unique protease present in saliva that specifically acts on one of the HIV proteins as a substrate.

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References


