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Involvement of tryptase-related cellular protease(s) in human immunodeficiency virus type 1 infection

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Trypstatin, a new cellular Kunitz-type protease inhibitor purified from rat mast cells, inhibited syncytium formation in human immunodeficiency virus type 1 (HIV-1)-infected CCRF-CEM and uninfected Molt-4 clone 8 at a concentration of 1 μM. Anti-rat tongue mast cell tryptase antibodies reacted with Molt-4 clone 8 cells, as determined by Western blot and by immunofluorescence. In addition, the antibody inhibited syncytium formation. These findings along with homologous sequences with trypstatin and a neutralizing epitope of gp120 of HIV-1 suggest that a tryptase-like cellular enzyme(s) is involved in HIV-1 infection.

HIV-1; Trypstatin; Neutralization; Tryptase

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndrome (AIDS), infects cells via interactions between cellular receptors (CD4 molecules) and the external envelope glycoprotein (gp120) [1,2]. The mechanism by which HIV-1 enters host cells after binding has, however, not yet been clarified. Previous studies showed that neutralization of endosomal compartments with lysosomotropic agents did not effectively inhibit HIV-1 nucleocapsid entry into the cytoplasm, and HIV-1 infection was observed even if the subunit of the CD4 molecule, necessary for internalization of the molecule, was absent as a result of genetic deletion [3,4]. These findings suggest that post-binding events of HIV-1 on cell surfaces are necessary for infection of cells by HIV-1. Experiments using synthetic peptides, encoding gp120, and a neutralizing monoclonal antibody clarified that the epitope consisting of 24 amino acids at positions 308-331 of gp120 is essential for infection by the HTLV-IIIB viral strain [5,6]. Furthermore, the epitope portion of gp120 is different from the domain of gp120 (amino acid residues 367-439), which plays a role in the binding of gp120 to CD4 molecules [7]. Successive studies clarified that the neutralizing epitope is homologous to the active sites of inter-α-trypsin inhibitor and human urinary trypsin inhibitor (UTI), and the synthetic peptide corresponding to the epitope inhibited trypsin activities. In addition, at concentrations ranging from 100 μM to 1 mM, UTI and the synthetic peptide inhibited syncytium formation caused by HIV-1-infected and uninfected T cell lines. These findings suggest that the neutralizing epitope might be accessible to an unknown cellular protease(s) upon infection (Koito, A., submitted). Recently, a new cellular Kunitz-type protease inhibitor, trypstatin, was purified from rat peritoneal mast cells and some of its amino acid sequences were also homologous with the neutralizing epitope [8,9]. Trypstatin inhibits tryptase activities in rat mast cells. In this

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communication, we have examined the effects of trypstatin and antibodies against tryptase on HIV-1 infection using HIV-1-susceptible T cell lines.

2. MATERIALS AND METHODS

2.1. *Purification of trypstatin and preparations of anti-tryptase antibodies*

Trypstatin and tryptase from rat peritoneal mast cells were purified by the methods of Kido et al. [8,10]. Tryptase from tongue and tryptase from lung were also purified from tongue and lung mast cells, respectively (Kido, H. and Katunuma, N., in preparation). These enzyme preparations and trypstatin appeared homogeneous after polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE). Antisera against tryptase from different tissues were obtained from rabbits (New Zealand Whites). Each purified protease was emulsified with an equal volume of Freund's complete adjuvant and was injected into rabbits intracutaneously at doses of 100 μg/rabbit. 3 weeks later, the rabbits were given subcutaneous weekly booster injections of 50 μg of the proteases with complete Freund's adjuvant for 3 weeks, and were bled 10 days after the last injection. Immunoglobulin G (IgG) (1.8 mg/ml) was prepared by the method of Arnon and Shapira [11]. Each antiserum stained granules of mast cells in the source tissue.

2.2. *Western blot*

Western blot was carried out as described previously [12]. The cell lysates obtained were fractionated by SDS-PAGE on a 12% acrylamide gel and were transferred to nitrocellulose. Individual nitrocellulose blots were treated with 1:200 rabbit antibodies against tryptase from various sources and with normal rabbit IgG. The second antibody used was biotinylated goat anti-rabbit antibody and bound antibodies were visualized by an enzyme immunoassay, using horseradish peroxidase-conjugated avidin as the indicating enzyme. Cell lines used were Molt-4 clone 8, which is highly susceptible and forms giant cells within several hours of cultivation with HIV-1-infected CCRF-CEM cells [13]. Another human T cell leukemia virus type I (HTLV-1)-infected cell line, called SKT-1B, was also used. This cell line has activated T cell features, is also susceptible to HIV-1 infection and dies after exposure to HIV-1 without forming syncytia [14].

2.3. *Cell surface staining*

Surface expression of tryptase was examined using the anti-tryptase antibody. Anti-tongue tryptase was chosen for this experiment, because the antibody reacted most strongly with both cell lines (Molt-4 clone 8 and SKT-1B) on Western blots. Normal peripheral blood mononuclear cells (PBMC) or concanavalin A (Con-A: Calbiochem Behring Corp., La Jolla, CA) -activated PBMC (5 μg/ml for 3 days) were also analysed for surface tryptase expression. One million cells from each line were incubated with 50 μl of 1:100 diluted rabbit anti-tongue tryptase antibody or normal rabbit IgG for 30 min on ice. The second antibody used was fluorescein isothiocyanate-conjugated goat F(ab')$_2$ anti-rabbit IgG (Tago Inc., Burlingame, CA). For the analysis of PBMC, stained cells were further reacted with phycoerythrin-conjugated Leu 4 (CD3) monoclonal antibody (Beckton and Dickinson Monoclonal Center, Mountain View, CA) to identify T cells. Stained cells were analysed by a laser flow cytometry (FACStar, Beckton and Dickinson Immunocytometry Systems, Mountain View, CA), using either single or two color analyses, respectively. Antigen-positive cells were expressed as a percentage of total T cells.

2.4. *Effects of trypstatin and anti-tongue tryptase antibody on syncytium formation*

HIV-1 (LAV-1) [15] -infected CCRF-CEM cells (2×10^4 cells per well) and Molt-4 clone 8 cells (1×10^5 cells per well) were washed twice with RPMI 1640 medium (Nissui, Tokyo) and co-cultured in 96-well microtiter plates (Falcon, Oxnard, CA) in the presence or absence of trypstatin or anti-tongue tryptase antibody. The cells were co-cultured in serum-free medium, ASF-104 (Ajinomoto, Tokyo) to avoid non-specific effects of protease(s) and protease inhibitors in fetal calf serum, in a humid atmosphere with 5% CO_2 at 37°C, and the plates were intermittently shaken. After 12 h of culture, syncytia formation was examined under an inverted microscope (magnification × 200).

(Protein)	(Number)															
Neutralizing Epitope	313	I	R	I	Q	R	G	P	G	R	A	F	V	T	T	G
Trypstatin	6	L	P	I	V	Q	G	P	C	R	A	F	A	E	L	L

Fig.1. Amino acid alignment of the neutralizing epitope of gp120 of HIV-1 and trypstatin. The numbers are from [6] and [8], respectively. A one-letter amino acid code is used and the box indicates homologous regions.

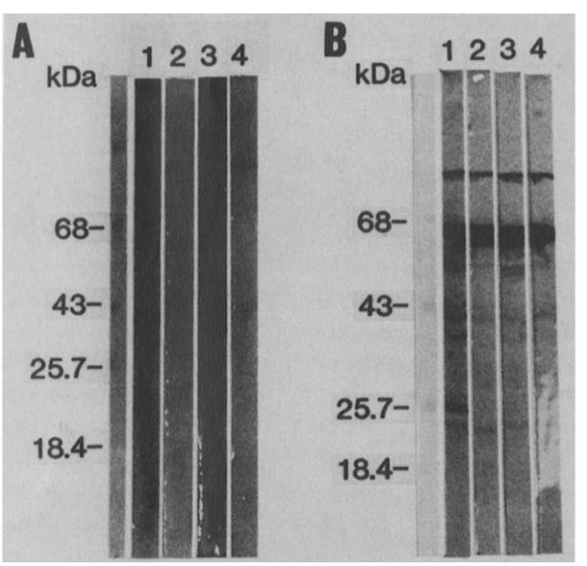

Fig.2. Western blot with anti-rat tryptase antibody. Cellular extracts of Molt-4 clone 8 (A) and SKT-1B (B) were used as antigens. Antibodies used were anti-tongue (lane 1), anti-lung (lane 2), anti-peritoneal (lane 3) mast cell tryptase and control rabbit IgG (lane 4).

Table 1

Cell surface staining with anti-tongue tryptase antibody. Relative proportion of antigen-positive cells

Cells	%
Molt-4 clone 8	96.6
Molt-4	87.0
SKT-1B	7.9
Resting T[a]	0.2
Activated T	7.8

[a] CD3-positive cells in non-activated PBMC

3. RESULTS

3.1. *Homologies of trypstatin and neutralizing epitope of HIV*

Fifty percent homology was found between amino acid residues 316 and 328, a part of the neutralizing epitope of HIV-1 [5,6] and residues 7–19 of trypstatin (fig.1). Interestingly, a highly conserved β-turn (GPGR) in the homologous regions is believed to be critical for antibody binding and type-specific virus neutralization [16].

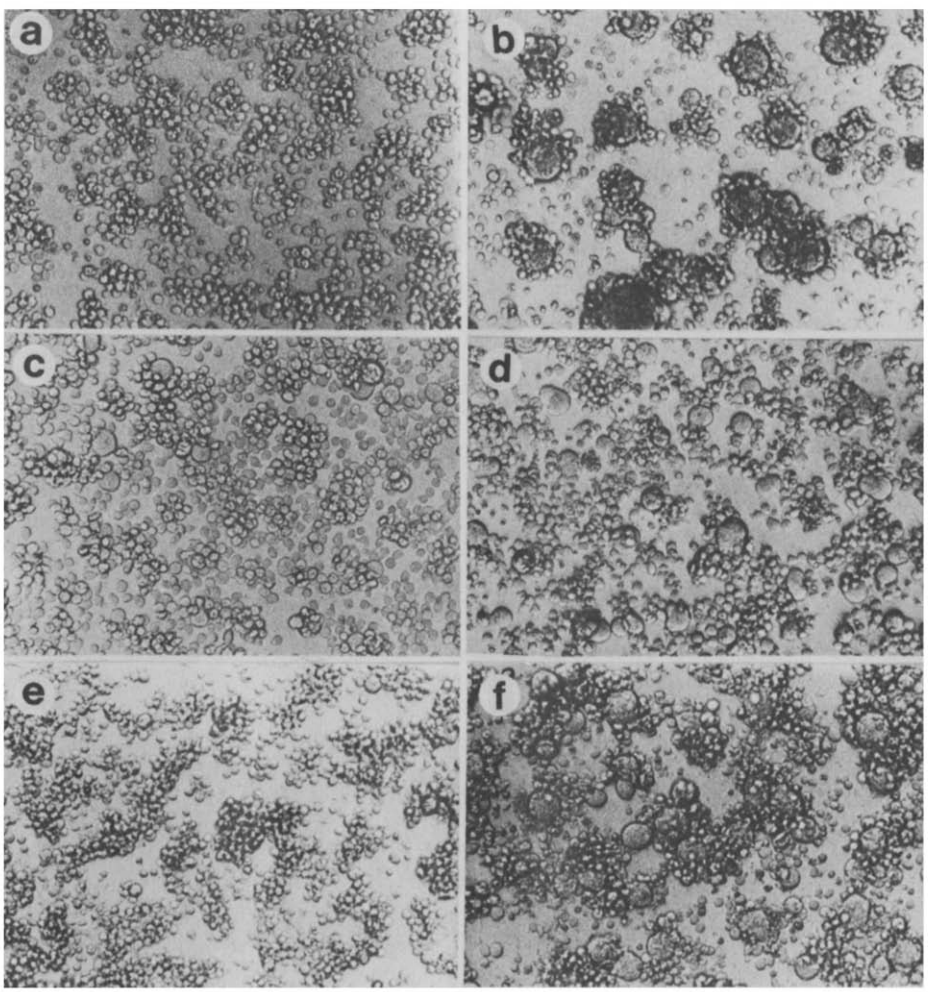

Fig.3. Effects of trypstatin and anti-tongue tryptase antibody on syncytium formation. (a) Molt-4 clone 8 alone (negative control); (b) LAV-1-infected CCRF-CEM and Molt-4 clone 8 (positive control); (c) trypstatin (1 μM); (d) trypstatin (300 nM); (e) rabbit anti-tongue tryptase antibody (600 μg/ml); (f) rabbit anti-tongue tryptase antibody (200 μg/ml).

In addition, the reactive site of trypstatin may be the R [14] and A [15] judging from the sequence homology [8].

3.2. *Western blot*

The Western blot was carried out using cellular extracts of Molt-4 clone 8 and SKT-1B [14] cells as antigens. The antibodies used included anti-tongue, anti-lung, anti-peritoneal mast cell tryptase antibodies and rabbit IgG as a control (fig.2). Control rabbit IgG bound non-specifically to proteins of 100 and 65 kDa in SKT-1B cells, and to a 100 kDa protein from Molt-4 clone 8 cells. However, anti-tongue and anti-peritoneal mast cell tryptase antibody recognized a 39 kDa protein in Molt-4 clone 8 cells, with the anti-tongue tryptase antibody staining this protein more clearly. However, anti-lung tryptase antibody did not react with Molt-4 clone 8 cells and SKT-1B cells. Anti-tongue tryptase antibody gave three bands, corresponding to 39, 36 and 25 kDa in SKT-1B cells. It is likely that the 39 kDa protein is important, and the 36 and 25 kDa proteins found in SKT-1B may be breakdown products of the 39 kDa protein.

3.3. *Antigen density of tryptase on cells*

Cellular tryptase antigen density was measured using the anti-tongue tryptase antibody (table 1). Around 90% of Molt-4 and Molt-4 clone 8 cells were anti-tongue tryptase antibody-positive. Before activation, less than 1% of PBMCs were double positive, that is CD3+, anti-tongue tryptase+, but increased to 7.8% after activation with Con-A. These findings suggest that T cells may express tryptase-like enzymes on cell surfaces after mitogen stimulation. Almost 8% of SKT-1B cells reacted with anti-tongue tryptase antibody, similar to activated T cells.

3.4. *Effects of trypstatin and anti-tongue tryptase antibody on syncytium formation*

The effects of trypstatin and anti-tongue tryptase antibody on syncytium formation caused by LAV-1-infected CCRF-CEM cells and Molt-4 clone 8 cells were examined under an inverted microscope. The formation of syncytia was inhibited completely by trypstatin at a concentration of 1 μM and the inhibition was also observed at a lower concentration of trypstatin (300 nM). Anti-tongue tryptase antibody at a concentration of 600 μg/ml also inhibited syncytium formation, but significant inhibition was not observed at the lower concentration of 200 μg/ml (fig.3).

4. DISCUSSION

Amino acid residues 308–331 of gp120 were found to be critical for neutralization of HIV-1 infection. The roles of this epitope in HIV-1 infection have not been clarified, but it is different from the site at which gp120 binds to CD4 molecules [5–7]. Our previous studies suggest that this portion may be nicked by cellular trypsin-type protease(s) or interacts with these protease(s) after binding of HIV-1 to the CD4 molecule, due to its homology to the reactive sites of the inter-α-trypsin inhibitor. In addition, human UTI, which is thought to be a proteolytic product of inter-α-trypsin inhibitor, inhibits syncytium formation at relatively high concentrations (1 mM–100 μM) (Koito, A; submitted).

In this report, we demonstrate marked inhibitory effects of HIV-1 infection by a new cellular protease inhibitor, trypstatin, derived from rat mast cells. The assumed reactive site of trypstatin also has high homology to the conserved core portion of the neutralizing epitope of gp120, as shown in fig.1. Also, trypstatin inhibits mast cell tryptase ($K_i = 3.6 \times 10^{-10}$ M), blood coagulation factor Xa ($K_i = 1.2 \times 10^{-10}$ M) and trypsin ($K_i = 1.4 \times 10^{-8}$ M) [8,9]. Trypstatin inhibited syncytium formation of co-cultured HIV-1-infected CCRF-CEM and Molt-4 clone 8 cells at a concentration as low as 1 μM. The concentration needed to inhibit syncytium formation in these cells by trypstatin is 100–1000-fold less than that required by UTI. These findings show that the effect of trypstatin is more specific than the serum and urinary inhibitors tested.

In addition, we found that anti-tongue tryptase antibody also inhibited the syncytium formation and recognized protein in the extracts of Molt-4 clone 8 and SKT-1B cells as demonstrated by Western blot. Immunofluorescent staining of these cells localized the antigen to the cell surface. In addition, mitogenic activation of PBMC induces expression of the antigen on T cells. The molecular mass of the immunoreactive antigen in both Molt-4 clone 8 and SKT-1B cells on SDS-PAGE was 39 kDa, which is larger than those of tryptase molecule from rat tongue and peritoneal mast cells, and

than that of rat lung tryptase molecule, which are 35 and 30 kDa, respectively. From these results, we speculate that Kunitz-type protease inhibitors, such as trypstatin and UTI, inhibit trypsin-type protease(s) on the surface of T cells, which play an essential role in HIV-1 infection. It has already been reported that cellular trypsin-type protease is involved in the maturation of HIV-1 gp160 to gp120 [17]. It is possible that trypstatin may inhibit this process. Alternatively, our findings strongly suggest that protease on the T cell surface, which is similar to rat tryptase, plays a role in successful HIV-1 infection of T cells by cleaving neutralizing epitope. The nature of the antigens recognized by anti-tongue tryptase antibody is at present unclear, and their characterization is now in progress.

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