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Short communication

Cystatin D, a natural salivary cysteine protease inhibitor, inhibits coronavirus replication at its physiologic concentration

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This study was conducted to examine the effect of cystatin D, a newly discovered salivary cysteine protease inhibitor, on human coronavirus replication. When MRC-5, human diploid lung cells, were incubated with dilutions of recombinant human cystatin D from 0.65–10 μM for 1 h prior to, during and after infection with coronavirus OC43 and 229e strains, a decrease in virus yield was observed resulting in an IC_{50} of 0.8 μM for both virus strains. This dose is within the normal concentration range of cystatin D, 0.12–1.9 μM found in saliva. When a single dose, 2.5 μM, was applied, cystatin inhibition of release of virus progeny was not overcome until three days post infection whereas inhibition by leupeptin, a serine and cysteine protease inhibitor, was completely abrogated by two days. When cellular toxicity was measured by ^{3}H-thymidine uptake, cystatin D did not markedly affect cell proliferation below a 10 μM dose. The results demonstrate that cystatin D is a potent inhibitor of coronavirus replication.

Key words: cystatins; human coronavirus; inhibition

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Cystatin D is the most recently described human cystatin that, in contrast to other family II cystatins, has a very restricted tissue distribution comprising only salivary and lachrymal glands (1, 3, 6). The normal concentration range of cystatin D in mixed saliva is 0.12–1.9 μM (6). It has been suggested that cystatin D in saliva might play a protective role against potentially harmful effects of proteinases of bacterial, fungal, viral and cellular origin and thus could be considered a component of the non-immune protective system in this cavity. Several investigators have obtained evidence for an antiviral effect on herpesvirus and coronavirus by cystatin C, the most widely distributed family II cystatin, with particularly high concentrations in seminal plasma and cerebrospinal fluid (1, 4, 5). Also, inhibition of poliovirus by chicken cystatin has been reported (10). Both coronavirus and poliovirus express viral cysteine proteinases that play important roles in replication by processing the polyproteins translated from the large open reading frames of these viruses (8, 10). Therefore, a direct effect of cystatin on the viral proteinases has been suggested (10), although the mechanism of cellular uptake of the inhibitor is unclear (4). We report here that recombinant human cystatin D inhibits human coronavirus replication, as was previously shown for cystatin C, and also that cystatin D is more effective than leupeptin in slowing the release of virus from infected cells.

Recombinant cystatin D was prepared using *Escherichia coli* expression vector p cystatin D-Arg containing a temperature-sensitive repressor gene, the phage -P_R promoter, an optimized ribosome-binding site, the *E. coli* outer membrane protein A signal peptide encoding sequence followed in frame by an Arg^{26}-cystatin D encoded cDNA, devoid of its signal sequence and the phage fd transcription terminator (3, 6). Isolation of Arg^{26}-cystatin D from periplasmic extracts was accomplished by immunosorption and gel filtration (3). Lyophilized, salt-free Arg^{26}-cystatin D was dissolved directly in Eagle's minimum essential medium and used at concentrations from 10–0.65 μM to treat duplicate monolayer cultures of MRC-5 cells (Viro-Med, Minnetonka, MN), about 140,000 cells per well, for 1 h before, during 1 h virus adsorption and during maintenance after infection in a yield reduction assay as described (5). Stock human coronavirus, strains OC43 and 229e prepared from supernatant medium of infected MRC-5

cells, was used at a multiplicity of infection of 1 in 0.2 ml to infect cultures. After adsorption at 37°C, the virus inoculum was removed by washing the cells twice. After incubation for 24 h at 33°C, infected cells were scraped into the medium and disrupted by freeze-thaw. The virus yield was titrated by plaque assay as described (5). Briefly, virus suspensions were diluted in Eagle's minimum essential medium and volumes of 0.2 ml were incubated with MRC-5 cell monolayers in 24-well trays (Costar, Cambridge MA) for 1 h at 37°C. The monolayers were overlaid with 0.5% agarose (Seakem, Rockland, ME) with Eagle's minimum essential medium and 2% fetal bovine serum. After 4 days at 33°C, plaques were stained with neutral red and counted. As shown in Fig. 1, cystatin D reduced the titer of human coronaviruses OC43 and 229e. The IC_{50} for both viruses was 0.8 μM. The IC_{50} compares favorably with that we obtained previously for cystatin C and leupeptin (5) and with that obtained for leupeptin by the plaque reduction assay of 229e virus (2). The IC_{50} is within the normal concentration range of cystatin D in saliva so that it could exert an inhibitory effect under normal circumstances. Coronaviruses can produce acute gastroenteritis in neonates and infants less than 12 months of age and cause diarrhea in HIV-infected patients. The oral route of infection is implicated in epidemic spread in children. Cystatin D may play a role in preventing salivary transmission in less contagious circumstances.

In order to compare the duration of inhibition of leupeptin and cystatin D, the inhibitors were added to cultures at 2.5 μM from 1 h before infection, as above, and supernatant medium was sampled daily for progeny virus. In Fig. 2, the inhibitory effect of leupeptin and cystatin D on release of virus progeny is shown. There was a 3.3 $\log_{10}$ reduction in released progeny at 24 h for cystatin D and a 1.2 $\log_{10}$ reduction at 48 h. For leupeptin, a 1.1 $\log_{10}$ reduction in released progeny was seen at 24 h and none at 48 h. When compared with the total yield method of assay (Fig. 1) greater inhibition of release of progeny virus was obtained for cystatin D than for leupeptin. This delay may be due to intracellular accumulation of virions or to release of aggregated or noninfectious virus progeny. Coronavirus infections are noncytolytic and virions are released from infected cells out of secretory vesicles that fuse with the plasma membrane. The 229e virus is not known to undergo proteolytic activation before infection nor are the RNA replicase proteins found in the virion as in the retroviruses where protease cleaves the *gag* and *gag-pol* precursor polyproteins into functional proteins of the mature virus particles (9, 11). Appleyard & Tisdale (2) failed to enhance infectivity or inactivate 229e virions by incubation with leupeptin. It is likely that more intracellular accumulation of virus particles is occurring in the presence of cystatin D. In contrast herpesviruses, which are also inhibited by cystatin, accumulate in the cytoplasm but are freed during virus-induced cytolysis (4, 7). The involvement of cystatin D in the normal proteolytic activity of the cell proteasome cannot be excluded (12).

The cytotoxicity of cystatin D was determined by measuring cell growth inhibition as determined by [^{3}H]-thymidine incorporation as previously described (5). MRC-5 cells, 2×10^5, in triplicate, were incubated with dilutions of inhibitor for 24 h at 37°C. [^{3}H]-Thymidine, 1 μCi per well, was added and at 36 h the cells were collected on glass fiber filters (934-AH), and radioactivity was determined in a scintillation counter (LS6800, Beckman, Irvine, CA). As shown in Fig. 3, inhibition of cell growth was minimal at inhibitor

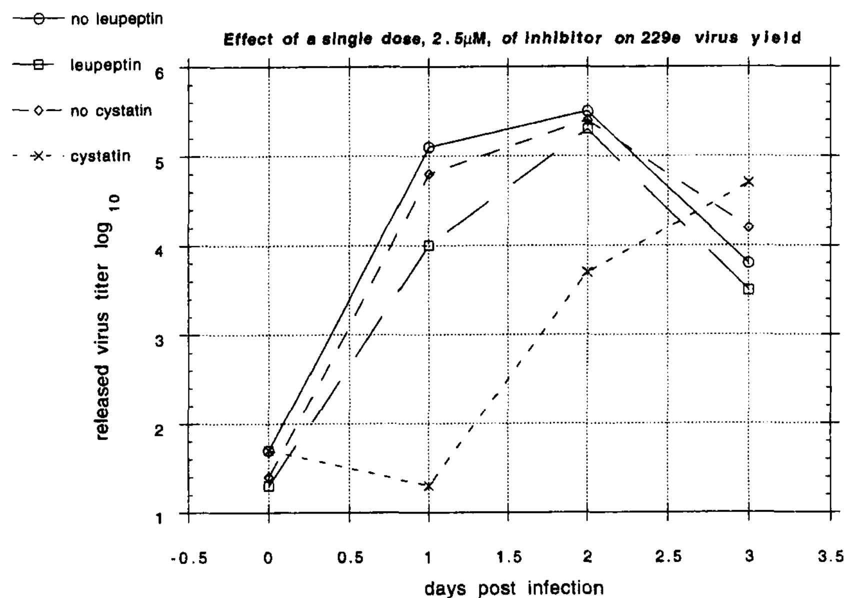

Fig. 2. Release of progeny virus over time from cells treated with 2.5 μM of inhibitors leupeptin and cystatin D in comparison with untreated infected cells

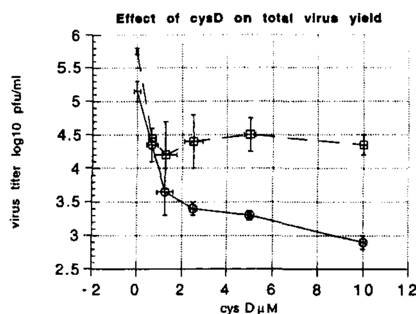

Fig. 1. Yield reduction curves showing the effect of cystatin D on replication of 229e virus (solid line) and OC43 virus (dashed line). Each value is the mean±SD from three independent experiments.

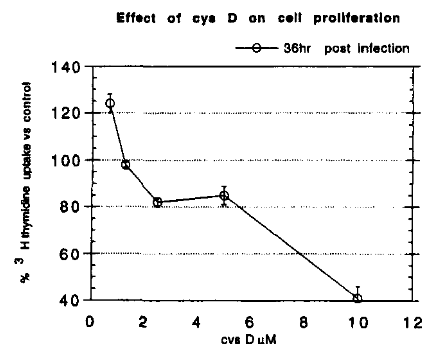

Fig. 3. Effect of cystatin D on [^{3}H]-thymidine incorporation in MRC-5 cells. The percentage of [^{3}H] incorporation ±SE at each concentration is shown.

concentrations of 5 μM and below. This suggests that the inhibitory effect on coronaviruses that occurred at concentrations below 10 μM was not due to general cytotoxic effects.

The family II cystatins, located on chromosome 20, include the so-called salivary cystatins S, SN and SA along with cystatins C and D. This group of proteinase inhibitors forms tight and reversible complexes with cysteine proteinases of the papain type such as cathepsins B, H, L, and S. Inhibitory activity resides in a wedge-shaped binding region formed by the *N*-terminal residues 8–10 (cystatin C numbering) and two loop forming segments jointly responsible for affinity for active site. Cystatin D is present only in saliva and tears. Unlike cystatin C, it does not inhibit cathepsin B and is less active against cathepsins L, H and S. Involvement of cystatin D with the cell secretory granules containing cathepsin B is not likely (13). We report here that cystatin D is as effective as cystatin C in inhibiting coronavirus replication. Also, cystatin D is apparently more effective than leupeptin in its duration of inhibition. Leupeptin exists in solution as an equilibrium between three species of which free aldehyde (2%) is the only form that can inhibit protease activity (13). Leupeptin binds to papain in two steps. First, inhibitor binds non-covalently to the active site of the protease. Second, the protease attacks the aldehyde carbon atom of the bound inhibitor to form a hemithioacetal transition state, which binds tightly. Leupeptin has a slower off-rate (K_d) than cystatin D, 10^{-9} *vs* 2.2×10^{-4} (3, 13). Cystatin D isolated from saliva displays a ragged N-terminus, probably due to proteolytic degradation during isolation (6). This may abort the activity of recombinant cystatin D if *N*-terminal residues 8-10 are lost. In further studies it will be important to define precisely the mechanism by which cystatins inhibit coronavirus replication. A classical papain-like cysteine protease is found as the leader protease (L-pro) of 229e virus along with a serine protease, similar to the 3C proteases of polioviruses (3CL-pro) (8). The direct effect of cystatin on the coronavirus papain-like protease is currently under investigation.

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