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Inhibitory Effects of Recombinant Human Cystatin C on Human Coronaviruses

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Cystatin C, a potent inhibitor of cysteine proteases such as papain and cathepsin B, was examined for its effect on human coronaviruses OC43 and 229e. Both viruses were >99% inhibited by 0.1 mM inhibitor. Endpoint titrations showed that inhibiting activity paralleled that of leupeptin, a serine and cysteine protease inhibitor, and indicated that 1 to 2 μM inhibitor, slightly above physiologic levels, was effective.

Cystatin C is a member of the cystatin superfamily of low-molecular-weight proteins which inhibit the activities of cysteine proteases and is present in all human biological fluids in concentrations implying a physiological importance of cystatin C as a controlling inhibitor of extracellular cysteine proteases (1). It consists of a single polypeptide chain of 120 amino acid residues. The N-terminal segment around Gly-11 is substratelike, and the N-terminal 11 amino acid residues are important for high-affinity binding between the inhibitor and a target proteinase (11). Inhibition of group A streptococci and herpes simplex virus type 1 by cystatin C and a tripeptide derivative, *N*-benzyloxycarbonyl-leucyl-valyl-glycine diazomethylketone (Z-LVG-CHN_2), prompted us to investigate whether human cystatin C possesses antiviral activity against the human coronaviruses OC43 and 229e (6, 7). These large, enveloped, plus-strand RNA viruses are important respiratory pathogens. Murine coronavirus infection can result in a high incidence of subacute and chronic demyelinating disease and is being studied as a model for multiple sclerosis (16).

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In this study we used recombinant cystatin C (rcystatin C) (pool 8) (molecular weight, 13,359) prepared from the cloned gene expressed in *Escherichia coli*. Native cystatin C and rcystatin C are indistinguishable in structure and display similar inhibition spectra and efficiencies (2). The lyophilized protein was reconstituted at 1.0 mg/ml in Eagle's minimum essential medium (EMEM) just prior to use and sterilized by filtration through a 0.22-nm-pore-size filter. Human coronavirus OC43 provided by G. Gerna, Pavia, Italy, was passaged 18 times in suckling mouse brain and adapted to African green monkey cells, to human lung cells (MA-321), and then to MRC-5 cells (ViroMed, Minnetonka, Minn.). Human coronavirus 229e was obtained from the American Type Culture Collection and passaged in MRC-5 cells in EMEM–5% fetal bovine serum. Murine coronavirus A59 propagated in DBT mouse neuroblastoma cells was obtained from L. S. Sturman, Albany, N.Y., and grown in Dulbecco's minimum essential medium with 10% fetal bovine serum.

In order to investigate the effect of rcystatin C on coronavirus replication, the inhibitor was tested at concentration of 0.1 mM, which was previously shown to result in >99.9% inhibition of herpes simplex virus without affecting cellular metabolism (7). Briefly, inhibitor was used to treat duplicate confluent cultures of MRC-5 cells (about 140,000 cells per well) for 1 h before infection, and the compound was added to the virus inoculum and to the maintenance medium after infection. The virus inoculum contained OC43 or 229e virus at a multiplicity of infection of 1 in 0.2 ml and 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 sonication and virus yield was titrated by plaque assay. For plaque assays, virus suspensions were diluted in EMEM and volumes of 0.2 ml were allowed to adsorb to MRC-5 or DBT cells in 24-well trays (Costar, Cambridge, Mass.) for 1 h at 37°C. The monolayers were then overlaid with 0.5 ml of 0.5% agarose with EMEM plus 0.2% serum. Cultures were stained with neutral red, and plaques were counted after 4 days at 33°C. As shown in Table 1, rcystatin C significantly reduced the replication of human coronaviruses OC43 and 229e but not the replication of murine coronavirus A59. The lack of effect of rcystatin C on murine coronavirus replication may be due to a lack of uptake of human rcystatin C in murine cells or possibly to the spread of MHV-A59 virus via fusion with the plasma membranes of uninfected cells. This mode of spread may be significant in that leupeptin, a serine and cysteine protease inhibitor, inhibits human coronavirus 229e but does not strongly inhibit murine coronavirus A59 and other coronaviruses, such as feline infectious peritonitis virus and porcine transmissible gastroenteritis virus, which undergo fusion with uninfected cell membranes during infection (4, 8). Cell fusion does not occur with human coronavirus OC43 (12, 17).

TABLE 1. Antiviral activities of rcystatin C

Virus	$\log_{10}$ titer of virus ($\bar{x}$ ± SE) in:		
	Medium	Medium with rcystatin C at:	
		0.1 mM	20 μM
OC43	5.1 ± 0.3	2.7 ± 0.4 (P = 0.003)[a]	3.1 ± 0.4 (P = 0.002)
229e	4.8 ± 0.2	2.1 ± 0.1 (P = 0.005)	2.9 ± 0.4 (P = 0.001)
A59	7.0 ± 0.1	6.8 ± 0.1	6.4 ± 0.1

[a] Results from triplicate experiments were analyzed by a paired t test; P values of ≤0.05 are considered significant.

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TABLE 2. Effect of rcystatin C and leupeptin on cell growth

Cell type	% of control cell [^{3}H]thymidine count ($\bar{x}$ ± SD)[a] with:							
	rcystatin C at (μM):				Leupeptin at (μM):			
	50	25	10	5	50	25	10	5
MRC-5	17 ± 5	58 ± 17	83 ± 8	96 ± 18	109 ± 29	95 ± 10	118 ± 15	97 ± 16
DBT	1 ± 0.3	64 ± 12	106 ± 9	106 ± 4	65 ± 9	62 ± 20	73 ± 35	95 ± 22

[a] Values are from three independent experiments.

To determine the range over which rcystatin C and leupeptin inhibit virus replication, leupeptin at concentrations from 200 to 0.3 μM and rcystatin C at concentrations from 100 to 0.1 μM were tested as described above. Leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal hemisulfate; molecular weight, 475.6; Sigma) was reconstituted in EMEM at 1,000 mg/ml and stored at −70°C (3). A comparison of inhibition of virus yield at increasing concentrations of inhibitor shows that nearly identical molar concentrations of leupeptin and cystatin C were required to achieve a yield reduction of >98% (Fig. 1). Inhibition of human coronavirus OC43 and 229e by rcystatin C was obtained at concentrations as low as 1 to 2 μM. Physiologic levels of cystatin C in serum and cerebrospinal fluid are 0.1 and 0.5 μM, respectively, but levels in serum are increased during uremia (1). In hereditary cystatin C amyloid angiopathy, the concentration of cystatin C in cerebrospinal fluid is abnormally low (1). This suggests that cystatin C may exert a protective effect against the spread of coronavirus infections.

The cytotoxic effect of rcystatin C in MRC-5 and DBT cells was minimal in that no cell rounding or detachment from the monolayer was observed at the concentrations used to inhibit virus replication. Cell growth inhibition by rcystatin C and leupeptin was compared by incubation of MRC-5 and DBT cells (2×10^5 per well, in triplicate) in EMEM alone or with various dilutions of inhibitor for 72 h at 37°C. [^{3}H]thymidine (1 μCi per well) was added at 56 h, the cells were collected on glass fiber filters (934-AH; Whatman) at 72 h, and radioactivity was determined in a scintillation counter (LS6800; Beckman, Irvine, Calif.). As shown in Table 2, inhibition of cell growth was minimal at inhibitor concentrations below 25 μM.

In order to characterize the effect of rcystatin C on viral replicative processes, the yield of virus following removal of rcystatin C at various times after infection with OC43 virus was examined. MRC-5 cells in 24-well trays were incubated with 15 μM rcystatin C in EMEM, beginning with the virus adsorption (multiplicity of infection of 1 for 1 h at 37°C) and continuing for various lengths of time during incubation at 37°C, which was followed by replacement with EMEM alone. After harvest of the infected cells at 24 h, virus yield was determined by plaque assay. The reversibility of the rcystatin C effect was tested by adding cycloheximide (10 mM) (Sigma), an inhibitor of protein synthesis at the level of translation, in combination with rcystatin C (15 μM), beginning with the virus adsorption period and continuing for various lengths of time during incubation at 37°C, which was followed by replacement with EMEM alone. Plaque assay of the virus yield at 24 h was performed.

As shown in Table 3, rcystatin C most effectively inhibited replication when present throughout virus replication. Moreover, rcystatin C inhibition in the presence of cycloheximide was reversible for up to 8 h after infection. From the control virus growth curve, we observed that the coronavirus inoculum was internalized and eclipsed by 2 h after initiation of infection. Similarly, internalized mouse coronavirus was not detected in either permissive cells or nonpermissive cells by 2 h after infection (13). When translation was blocked by cycloheximide, removal of both inhibitors then allowed viral mRNA which had penetrated into the cytoplasm to be translated. Other events in uncoating, such as viral nucleocapsid dephosphorylation, would have occurred normally in infected MRC-5 cells treated with both inhibitors (5). These results indicated that rcystatin C was active beyond the penetration step during virus replication.

It is likely that papainlike proteases predicted to be part of the polymerase complex of coronaviruses would be inhibited by rcystatin C, particularly since the inhibitory activity of rcystatin C is 10-fold more effective against papain than against cathepsin B, a lysosomal enzyme (1). The polymerase gene (open reading frame 1) of murine and avian coronaviruses contains two overlapping large open reading frames. Sequence data suggest that open reading frame 1a contains a picornavirus 3C-like protease domain and two papainlike protease domains (10, 15). At least one enzymatic activity, an N-terminal autoprotease (p28), has been associated with the murine coronavirus A59 gene 1 product by in vitro translation, and in the presence of leupeptin protease inhibitor, the synthesis of p28 was diminished (9). Our results are consistent with the postulate that inhibitors of cysteine proteases may regulate the functions of the polymerase gene products. Likewise, chicken cystatin could partially block poliovirus protein cleavages (14). Further

TABLE 3. Effect of removal of rcystatin C and cycloheximide at various times after infection with OC43[a]

Inhibitor	Range of inhibition (%) of titer[b] at:					
	0 h	2 h	4 h	6 h	8 h	24 h
rcystatin C	93–97	88–95	93–99	100	100	100
rcystatin C + cycloheximide	63–78	0–10	69–80	86–94	95–99	100
Cycloheximide	—	—	85–89	87–99	—	—

[a] In the growth curve for the virus control, titers of samples taken at the times indicated were 10^2, $<10^2$, $10^{2.3}$, $10^{2.9}$, $10^{3.7}$, and $10^{4.6}$ PFU/ml, respectively. Inhibitors were removed at the hours indicated after virus adsorption.

[b] Percentages are averages from duplicate determinations. —, not done.

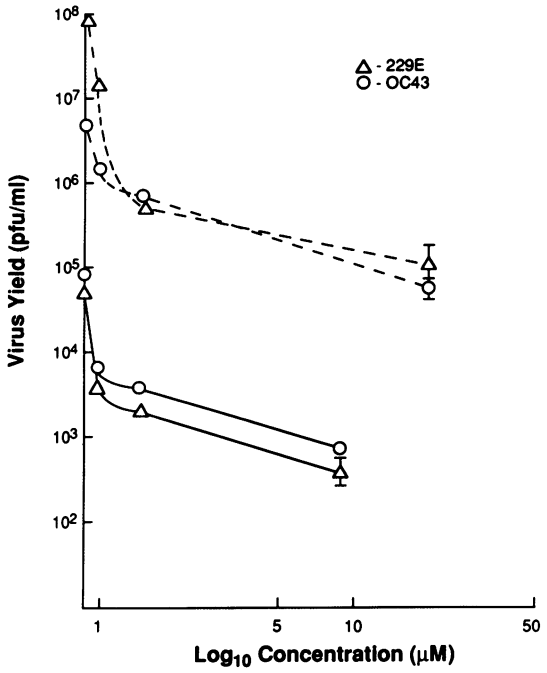

FIG. 1. Yield reduction curves showing the effects of rcystatin C (solid lines) and leupeptin (dashed lines) on the yield of OC43 and 229e coronaviruses. Each value is the mean ± standard deviation from three independent experiments.

characterization of the polymerase gene products of coronaviruses is required to address this question.

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