Cystatin C, a Human Proteinase Inhibitor, Blocks Replication of Herpes Simplex Virus

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Cystatin C is a human cysteine proteinase inhibitor present in extracellular fluids. Cystatin C and a tripeptide derivative (Z-LVG-CHN₂) that mimics its proteinase-binding center, were tested for possible antiviral activity against herpes simplex virus type 1 (HSV) and poliovirus type 1. Both recombinant cystatin C and Z-LVG-CHN₂ displayed strong inhibitory effects on HSV replication, whereas no significant effect on poliovirus replication was seen. The molar concentration of cystatin C that gave total inhibition of HSV replication was lower than that of either Z-LVG-CHN₂ or of acyclovir, the drug currently most used against HSV infections. These results suggest that cysteine proteinase inhibitors might play a physiological role as inhibitors of viral replication and that such proteinase inhibitors, or peptide derivatives that mimic their proteinase-binding centers, might be used as antiviral agents.

Cysteine proteinases participate in the intracellular catabolism of proteins and peptides of eucaryotic cells (11) and are also assumed to be involved in the proteolytic processing of prohormones (15) and proenzymes (17) in such cells. The physiological activities of cysteine proteinases are controlled by a set of protein inhibitors which constitute the cystatin superfamily of proteins (6). The probable proteinase-binding center of one of the most important human cystatins, cystatin C, has recently been identified (4) and a small peptide derivative that mimics part of this region and carries a sulfhydryl-reactive group (9) has been shown to be an irreversible cysteine proteinase inhibitor (8). This derivative, N-benzoyloxycarbonyl-leucyl-valyl-glycine diazomethylketone (Z-LVG-CHN₂), was tested for antibacterial activity and found to block the growth of group A streptococci both in vivo and in vitro, apparently by inhibiting a cysteine proteinase specific for these bacteria (8). Cysteine proteinases have been reported to participate in viral replication by proteolytic processing of large precursor polyproteins (7, 14, 16), and a report that chicken cystatin could partially block poliovirus replication (12) prompted us to investigate whether Z-LVG-CHN₂ possesses not only antibacterial but also antiviral activity.

In a first set of experiments, paper disks were impregnated with Z-LVG-CHN₂ (24 µg per disk) and placed on monolayers of green monkey kidney cells infected with poliovirus type 1 or herpes simplex virus type 1 (HSV). In this screening for antiviral activity, no inhibition of plaque formation was observed with poliovirus. However, marked inhibition zones were obtained around the disks when the cells were infected with HSV. We then analyzed whether addition of Z-LVG-CHN₂ or human recombinant cystatin C (2) to the medium could influence poliovirus or HSV growth in cell cultures. In these experiments (Table 1), green monkey kidney cells (strain GMK-AH1) were infected with poliovirus (Mahoney strain) or HSV (F strain) at multiplicities of 1 and 10, respectively. After 2 h of attachment, serum-free medium (Flow Laboratories, Inc.) was adjusted to contain 0.10 mM cystatin C or 0.40 mM Z-LVG-CHN₂. Z-LVG-CHN₂ was dissolved in serum-free medium containing 1% dimethyl sulfoxide to keep the peptide derivative in solution. Cells were incubated for 48 h at 37°C, followed by freezing and thawing. Plaque titrations were performed on GMK-AH1 cells as previously described (10). In all assays, three petri dishes (Falcon; 6-cm diameter) were used per dilution step. Values were based on counts of at least 40 PFU, and the values in Table 1 are from typical and reproducible experiments. Also in these experiments, HSV replication was blocked (>99.9% inhibition) by Z-LVG-CHN₂, whereas the effect on poliovirus replication was hardly significant. Moreover, human recombinant cystatin C was found to be a potent inhibitor of HSV replication but with no influence on poliovirus replication (Table 1). Although other mechanisms of action cannot be completely excluded, the most logical and plausible way of explaining this pronounced anti-HSV activity is to assume that cystatin C and Z-LVG-CHN₂ interfere with a cysteine proteinase(s) of importance to HSV replication. The finding that human cystatin C, in contrast to chicken cystatin (12), does not suppress poliovirus replication, was unexpected, since these cystatins display 60% sequence homology. However, it is known that the proteinase-inhibitory capacities of the two cystatins differ (5), and it is thus conceivable that they might also differ in their capacities to inhibit certain viral cysteine proteinases crucial for replication.

The anti-HSV potencies of cystatin C and Z-LVG-CHN₂ were compared with each other and with that of acyclovir (Wellcome Diagnostics) by investigation of dose-response curves. In the experiments presented in Fig. 1, GMK-AH1 cells were inoculated with HSV (10 PFU per cell, corresponding to 5 × 10⁶ PFU/ml) for 2 h at 37°C. After washing,

| Table 1. Antiviral activities of cystatin C and Z-LVG-CHN₂. |
|---------------------------------|-----------------|-----------------|-----------------|
| Virus                           | PFU/ml in:      |                 |                 |
|                                 | Medium          | Cystatin C      | Medium with 1%  | Z-LVG-CHN₂, in |
|                                 |                 | with 1% DMSO    |                 | 1% DMSO        |
| Poliovirus type 1               | 1.2 × 10⁶       | 1.1 × 10⁶       | 1.5 × 10⁶       | 4.8 × 10⁷      |
| HSV                             | 8.8 × 10⁶       | 2.4 × 10⁶       | 6.4 × 10⁶       | 1.4 × 10³      |

* DMSO, Dimethyl sulfoxide.

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a residual infectivity of about 10^2 PFU was recorded, and this value thus represents the zero-time background levels of the growth curves. At this time point, serum-free medium containing different concentrations of the inhibitors was added to the HSV-infected cells, followed by incubation at 37°C for 48 h. Finally, the cells were frozen and thawed and plaque titrations were performed (10). The concentration of cystatin C required for inhibition of viral replication to background levels was about 0.13 mM, while the corresponding concentrations for Z-LVG-CHN2 and acyclovir were 0.44 and 0.22 mM, respectively. Thus, the anti-HSV potencies of both native cystatin C and the peptide derivative Z-LVG-CHN2, which mimics part of its proteinase-binding center, are of the same order of magnitude as that of acyclovir. If we assume that the anti-HSV effects of both cystatin C and Z-LVG-CHN2 are mediated by their inhibition of a cysteine proteinase crucial to HSV replication and that the accessibility of this proteinase for both inhibitors is equal, the fourfold difference between the potencies of the inhibitors might be explained by more efficient inhibition of the putative proteinase by cystatin C. Thus, Z-LVG-CHN2 mimics only part of the proteinase-binding center of cystatin C (4, 8), and work in progress has indeed shown that Z-LVG-CHN2 displays an inhibition spectrum and efficiency different from those of native cystatin C.

In the experiments described above, cystatin C and Z-LVG-CHN2 were added to the medium after the cells had been incubated with the viruses and then carefully washed. As the inhibitors were not present during this first step, they seem to interfere with later events of importance to HSV replication. This was also demonstrated when Z-LVG-CHN2 (0.40 mM) was mixed with a diluted HSV suspension and present only during the 2 h of viral adsorption to the cells. In this case, the number of plaques was not reduced. Finally, inhibition of HSV replication was not restricted to monkey kidney cells. Thus, although HSV replication was less efficient (yield, around 10^2 PFU/ml) in a human rhabdomyosarcoma cell line (RD), it was completely blocked (>99.9% inhibition) by Z-LVG-CHN2.

Cystatin C is present mainly in extracellular body fluids (1, 3), whereas most cysteine proteinases are involved mainly in intracellular physiological processes. It has therefore been generally assumed that the biological function of cystatin C is to inhibit and control cysteine proteinases which appear in extracellular fluids as a result of pathophysiological events. Another interesting possibility is that cystatin C might enter the membranes of which have been damaged by viral attack, complement activation, malignant transformation, etc., and then inhibit intracellular cysteine proteinases with vital functions. We have not identified the putative target proteinase(s) for cystatin C or Z-LVG-CHN2 in HSV-infected cells, and it is not clear from the literature whether HSV itself and/or the infected cells encode cysteine proteinases required for HSV replication. However, interference with any proteolytic event of crucial importance to HSV replication can explain the antiviral effect of cystatin C and Z-LVG-CHN2. The pronounced effect on HSV replication is in contrast to the results obtained with poliovirus. Thus, although the same eucaryotic cells and the same concentrations of cystatin C and Z-LVG-CHN2 were used, poliovirus replication was not significantly influenced. These results strongly indicate that cystatin C and Z-LVG-CHN2 do not block HSV replication by displaying general cytotoxic effects.

The data presented in this report concerning human recombinant cystatin C, Z-LVG-CHN2, and their influence on HSV replication support the concept that proteinase inhibitors might be used more generally as antiviral agents by inhibiting proteinases required for viral replication (13). Since large amounts of both of these inhibitors can be obtained, it should be possible to use them to isolate their target proteinase(s) by affinity chromatography, for more extended screening of antiviral activity, and for studies of their effects on experimental infections in animals. In this context, it is also encouraging that large doses of Z-LVG-CHN2 have been found to be nontoxic to mice (8).

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