The cysteine protease inhibitors cystatins inhibit herpes simplex virus type 1-induced apoptosis and virus yield in HEp-2 cells

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The role of cystatins in herpes simplex virus (HSV)-induced apoptosis and viral replication has been studied. Human epithelial (HEp-2) cells infected with wild-type HSV-1 (F), with a deletion virus lacking the anti-apoptotic gene Us3 (R7041) or with a deletion virus lacking the anti-apoptotic genes Us3 and ICP4 (d120) were treated with cystatin A, C or D. Cells and culture media were studied at different time points for replicating HSV-1 and for apoptosis. Cystatins C and D inhibited the yield of replicative HSV-1 significantly in HEp-2 cells. In addition, cystatin D inhibited R7041 and d120 virus-induced apoptosis. Moreover, cystatin A inhibited R7041-induced apoptosis. These inhibitory effects of cystatins on virus replication and apoptosis are likely to be separate functions. Cystatin D treatment decreased cellular cathepsin B activity in HSV-1 infection, suggesting that cathepsin B is involved in virus-induced apoptosis.

The cysteine protease inhibitors cystatins inhibit papain-like cysteine proteases (Järvinen & Hopsu-Havu, 1975; Järvinen & Rinne, 1982; Green et al., 1984). Cystatins are divided into three types according to their structural details and their distribution in the body. Twelve functional cystatins have been characterized in humans thus far. Cystatin A (type 1) was isolated from rat skin (Järvinen & Hopsu-Havu, 1975). It is expressed abundantly in the squamous epithelium of tissues (Rinne et al., 1978; Kominami et al., 1984). Thus, cystatin A is found in tissues protecting against exogenous factors (bacteria and viruses). In contrast, cystatin C (type 2) is the dominating inhibitor of extracellular fluids (Abrahamson et al., 1986). Cystatin D (type 2) is excreted in saliva and tears (Freije et al., 1993). In addition to their other biological functions, cystatins have been shown to inhibit RNA virus-induced programmed cell death (Björklund et al., 1997). Moreover, the antiviral properties of cystatins (Björck et al., 1990; Aoki et al., 1995; Gu et al., 1995; Collins & Grubb, 1998) suggest that they may also inhibit cysteine proteases used by viruses during infection.

Herpes simplex virus type 1 (HSV-1) is an enveloped DNA virus with a large genome. The replication cycle is 18 h in epithelial cells. A productive viral infection is accompanied by inevitable host-cell destruction. HSV-1 exerts both pro- and anti-apoptotic effects. HSV-1 induces apoptosis by 1 h post-infection and the first apoptosis-blocking proteins are synthesized between 3 and 6 h, in the period termed the ‘prevention window’ (Aubert & Blaho, 1999). This period corresponds to the transition from the immediate-early to the early phase of viral gene expression. For example, infection with HSV-1 lacking either immediate-early ICP27 or ICP4 proteins results in apoptosis (Aubert & Blaho, 1999; Leopardi & Roizman, 1996). The late protein kinase Us3 blocks virus-induced apoptosis (Leopardi et al., 1997) and has been shown to block caspase-dependent double cleavage and activation of the pro-apoptotic BAD (Benetti et al., 2003). Other HSV-1 proteins reported to participate in blocking apoptosis are the viral glycoprotein gD (Zhou et al., 2000), immediate-early regulator ICP22 (Aubert et al., 1999) and the late Us5 glycoprotein (Jerome et al., 1999).

In this report, we have studied the influence of one intracellular (cystatin A) and two extracellular (cystatin C and D) cystatins on HSV-1 replication and on HSV-1-induced apoptosis in a human epithelial cell line (HEp-2) infected with either wild-type virus or HSV-1 viruses lacking the anti-apoptotic genes Us3 or ICP4.

Cystatin A was purified from human post-mortem spleen according to the method of Järvinen & Hopsu-Havu (1975) at the Department of Forensic Medicine, University of Turku, Finland. Its purity was confirmed by gel electrophoresis and immunoblotting using an anti-cystatin A...
antibody. The preparation was free of other studied cystatins. Cystatins C and D were produced by expression in Escherichia coli as described previously (Abrahamson et al., 1988; Freije et al., 1993).

In order to study HSV-1 replication in cystatin-treated HEp-2 cells, the cells were infected with wild-type HSV-1 (F), the Us3 protein kinase-deletion virus R7041, its rescue virus R7306 or Us3- and ICP4-deletion virus d120 at an m.o.i. of 5 or 0.05. The R7041 and R7306 viruses were generously provided by Dr Bernard Roizman (University of Chicago, IL, USA) and the d120 virus was a kind gift from Dr Neal DeLuca (University of Pittsburgh, PA, USA). Virus stocks were grown in Vero cells obtained from the ATCC or in Vero E5 cells provided by Dr N. DeLuca. The virus titres from the culture supernatants were analysed and the obtained stock titres were between $5 \times 10^8$ and $1 \times 10^9$ p.f.u. ml$^{-1}$. For some experiments, the stock of HSV-1 (F) was inactivated by UV light for 30 min (mock). Loss of infectivity was verified by plaque assays in cell cultures. The decrease in infectivity of the mock preparation was more than 10 000-fold. The human epithelial cell line HEp-2 was originally obtained from the ATCC.

We first tested different time points for addition of cystatins. The time point of 1 h post-infection (p.i.) was selected for further studies to exclude the interference of cystatins with virus–cell-receptor binding or the first virus–internalization steps. Different concentrations of cystatins were also tested. The optimal concentration for cystatin A was 125 nM and, for cystatin C or D, it was 100 nM. First, we detected replicative virus in the infected cell monolayers at a time point of 21 h p.i. by the rapid-culture method as described previously (Ziegler et al., 1988). In R7041-infected HEp-2 cells, cystatins C and D reduced the formation of viral plaques significantly ($P=0.006$ and 0.001, respectively) (Fig. 1a). In HSV-1 (F)-infected cells, no significant difference between untreated or cystatin-treated cells was seen (data not shown).

In order to study whether cystatins have an effect on the release of HSV-1 into the culture medium, the amount of infectious virus was measured from the culture medium collected from infected HEp-2 cell cultures. Cystatins C and D reduced the release of the infectious R7041 virus significantly ($P=0.026$ and 0.005, respectively) (Fig. 1b). Moreover, cystatins A, C and D reduced the release of infectious HSV-1 (F) into the culture medium significantly ($P=0.010$, 0.002 and 0.002, respectively). d120-infected cells were not studied for virus yield or replication, because the d120 virus is unable to replicate in HEp-2 cells.

To study the anti-apoptotic effects of cystatins, HEp-2 cells were infected with d120, R7041, R7306 or HSV-1 (F) viruses, and apoptosis was measured by Hoechst staining (Fig. 2). At an early time point of infection (6 h p.i.), no significant increase in the number of apoptotic cells was seen (data not shown). However, the number of apoptotic cells increased over the course of time in d120- and R7041-infected cell cultures. The level of apoptosis was at its highest in d120-infected cells at 16 h p.i. (approx. 25 %) and in R7041-infected cells at 21 h p.i. (approx. 15 %; data not shown).

**Fig. 1.** Cystatin treatment reduced HSV-1 replication and yield in infected HEp-2 cells. (a) HEp-2 cells infected with R7041 at an m.o.i. of 0.05 were treated with cystatin A, C or D and the infected monolayers of cells were immunostained for HSV-1 gC antigen at 21 h p.i. The number of HSV-1 plaques was reduced significantly in cystatin C- and D-treated cells compared with untreated cells ($P=0.006$ and 0.001, respectively). Data are means ± SD of the relative proportion of p.f.u. in comparison to no cystatin treatment (untreated). Experiments were repeated at least twice. (b) Release of infectious virus was analysed by a rapid-culture method collected from infected HEp-2 cells. Tenfold dilutions of the medium were distributed on Vero cells and immunostained for HSV-1 after 18 h incubation. The difference between untreated and cystatin C- or D-treated cells was significant in R7041-infected cells ($P=0.026$ and 0.005, respectively) and in HSV-1 (F)-infected cells ($P=0.002$ and 0.002, respectively). In addition, in HSV-1 (F)-infected HEp-2 cells, cystatin A reduced the release of infectious virus ($P=0.010$). Data are means ± SD of the relative proportion of p.f.u. in comparison to no cystatin treatment (untreated). Experiments were repeated at least twice. *$P<0.05$; **$P<0.005$; ***$P<0.001$. **P. Peri and others**
In accordance with these results, the effect of cystatins on virus-induced apoptosis was studied at 16 h p.i. in d120-infected cells and at 21 h p.i. in R7041-, F- or mock-infected cell cultures. Treatment with cystatin A or D decreased the number of apoptotic cells in R7041-infected cell cultures significantly \( (P < 0.018 \) and \( 0.022, \) respectively) \( (\text{Fig. 2a}) \). In addition, cystatin C diminished the number of apoptotic cells in R7041 infection \( (P = 0.068) \). In d120-infected cells, cystatin D treatment reduced the number of apoptotic cells significantly \( (P = 0.038) \) and both cystatins A and C lowered the level of apoptosis slightly \( (\text{Fig. 2a}) \). There were 6.5 % apoptotic cells in HSV-1 (F) infection, 4.5 % in the mock-infected cells and 4.0 % in uninfected cells. The R7306 virus yielded similar results to HSV-1 (F) \( (\text{data not shown}) \).

In order to look for the anti-apoptotic mechanisms of cystatins, we studied their effects on activity of cathepsins. For cathepsin B and L assays, HEp-2 cells were plated on 96-well culture plates, grown to 80 % confluence and infected with d120, R7041 or F at an m.o.i. of 5. Cells were treated with cystatin A, C or D at 1 h p.i. and cathepsin activities were analysed at 5 and 21 h p.i. \( (\text{data not shown}) \). The findings were most evident at 21 h p.i. Cathepsin B activity was reduced significantly at 21 h p.i. in d120-, R7041- and F-infected cells with cystatin D treatment \( (P = 0.001, 0.002 \) and \( 0.020, \) respectively) \( (\text{Fig. 3}) \). In addition, cystatin C reduced the activity of cathepsin B in d120- and HSV-1 (F)-infected cells significantly at 21 h p.i. \( (P = 0.002 \) and \( 0.010, \) respectively). No significant difference in cathepsin L activity was seen between untreated and cystatin A-, C- or D-treated cells \( (\text{data not shown}) \).

Statistical analyses were performed with SAS software by using the Dunnett generalized linear model procedure. Values from cystatin A-, C- or D-treated cells were compared pairwise with corresponding values of untreated cells. Values of \( P < 0.05 \) were considered statistically significant.
Cystatins have been proposed to have antiviral activities. Bjo¨rck et al. (1990) showed that human recombinant cystatin C inhibited HSV-1 replication in green monkey kidney cells. In addition, proline-rich proteins and salivary cystatins were able to inhibit HSV-1 replication (Gu et al., 1995). In contrast, the level of cystatins in saliva did not correlate with HSV-neutralizing activity (Va¨limaa et al., 2002). The target for cystatins in HSV-1 infection is still unclear. Recently, Kattenhorn et al. (2005) discovered a novel HSV-1 ubiquitin-specific cysteine protease encoded approximately within the 500 N-terminal residues of the UL36 gene product (UL36USP). UL36USP is activated late during viral replication and it is assumed that the ubiquitination of other virus proteins by UL36USP is needed for virion assembly, although at the time of UL36USP activation, most of the structural proteins are synthesized and virion assembly is nearly completed (reviewed by Mettenleiter, 2004). In addition, UL36USP is thought to have a role in virus egress from the cell. This finding of an HSV-1 cysteine protease could explain the inhibitory effects of cystatins on HSV replication. Cystatins evidently inhibited the release of virus into the cell-culture medium, suggesting that cystatins may interfere with the egress of virus from the cell.

Recent data suggest that cathepsins, the target proteins of cystatins, may act as mediators of apoptosis (Vancompernolle et al., 1998; Yamashima et al., 1998). The pro-apoptotic Bcl-2 family member Bid is cleaved by lysosomal proteases in an in vitro system and it can potently trigger cytochrome c release from mitochondria (Stoka et al., 2001). Other pro-apoptotic proteins, such as the Bcl-2 family member BAD, may also be cathepsin substrates.

HSV-1 prevents host-cell apoptosis by using several of its functions (Roizman & Knipe, 2001). For example, the Us3 protein kinase blocks apoptosis induced by the virus. Cartier et al. (2003) showed that Us3 prevents the cleavage of pro-apoptotic Bid by granzyme B and that the expression of Us3 is associated with the inhibition of caspase activation. Thus, a Us3-deletion virus is not able to prevent virus-induced apoptosis completely in a host cell.

In the present study, d120 and R7041 viruses induced apoptosis in HEP-2 cells. When cystatins were added to the cell culture, virus-induced apoptosis was partially inhibited. Although the mechanisms of inhibition of Us3-deletion virus-induced apoptosis by cystatins must be studied in more detail, it would be tempting to speculate that the prevention of apoptosis by cystatin D is due to inhibition of cathepsin B, and thus inhibition of the Bid-mediated apoptosis signalling cascade. To our knowledge, this is the first report on the regulation of cathepsin activity by cystatins in virus-induced apoptosis. The inhibitory...
effects of cystatins on virus replication and apoptosis are probably separate functions, because the release of wild-type HSV-1, showing minimal apoptosis, was reduced.

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References


