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# Rhabdovirus-Induced Apoptosis in a Fish Cell Line Is Inhibited by a Human Endogenous Acid Cysteine Proteinase Inhibitor

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**To determine the mechanisms of cell death in rhabdovirus-infected cells, we studied the infection of the epithelial papilloma of carp cell line with spring viremia of carp virus. Studies using electron microscopy, confocal microscopy, and agarose gel electrophoresis revealed changes in cell morphology and DNA fragmentation indicative of apoptosis. The virus-induced apoptosis was inhibited in cells treated with a human endogenous acid cysteine proteinase inhibitor.**

Recent studies have shown that apoptosis may play on important role in many viral infections (9–11, 15, 19, 20, 23, 25, 28, 29). Cell death due to rhabdovirus infection has been considered to result from necrosis following cell membrane damage caused by the budding virions (31). Two recent reports have, however, shown evidence for apoptotic cell death in vesicular stomatitis virus-infected HeLa cells and in rabies virus-infected mouse thymocytes (16, 18). The fish-pathogenic rhabdovirus spring viremia of carp virus (SVCV) is the causative agent of lethal hemorrhagic swim bladder inflammation and peritonitis in carp and many other fish species (31). In this report, we present evidence that apoptosis is involved in the cell death caused by SVCV in the epithelial papilloma of carp (EPC) cell line. The endogenous acid cysteine proteinase inhibitor (ACPI; also termed cystatin A), isolated from human palatine tonsils, inhibited SVCV-induced apoptosis in EPC cells without reducing the growth of the virus.

EPC cells were grown as monolayers in 24-well tissue culture plates at 20°C in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. The cells were infected with SVCV (ATCC VR-1390) at a multiplicity of infection of 0.1. At various times after infection, infected and uninfected cells were fixed in 3% glutaraldehyde and then treated with 1% osmium tetroxide in 0.15 M sodium cacodylate. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with a JEOL JEM-100SX transmission electron microscope. For studies on the morphology of cell nuclei, EPC cells were grown on glass coverslips. Infected and uninfected cells were fixed in 70% ethanol, treated with RNase, and stained with propidium iodide (50 μg/ml). The stained DNA was viewed under a Leica TCS confocal scanning microscope.

A virus-induced cytopathic effect (CPE) was first apparent 36 h after infection as focal areas which subsequently enlarged throughout the entire monolayer. The focal areas of SVCV-infected cells, exhibiting typical CPE, showed ultrastructural changes consistent with apoptosis (Fig. 1). The earliest ultrastructural changes in virus-infected cell cultures involved a reduction of cell volume, protrusions of the cell surface (blebbing), and breakdown of cells into membrane-bound bodies (Fig. 1B). This stage was rapidly followed by a further condensation of the cells with a vacuolization of the cytoplasm and concentration of the chromatin into one or several dense bodies (Fig. 1C). These condensed cells were frequently phagocytized by apparently normal EPC cells (Fig. 1D). The mitochondria in the condensed cells appeared to be of normal size. When stained with propidium iodide, the SVCV-infected EPC cells showed prominent condensation and fragmentation of the cell nuclei (Fig. 2), confirming the ultrastructural observations.

The viability of the virus-infected cells was assessed by the trypan blue exclusion assay, and the virus titers were determined by plaque assay. At 75 h after infection, when the virus titer had reached a maximum ($6.6 \times 10^7$ PFU/ml) in SVCV-infected cells and more than 70% of the cells appeared apoptotic, 72.0% ± 6.6% (mean ± standard deviation of 12 independent counts on 100 cells each) of SVCV-infected EPC cells still excluded trypan blue, suggesting that a large majority of the shrunken cells maintained their membrane integrity.

For studying DNA fragmentation, $10^5$ cells were lysed at intervals, ranging from 1 to 96 h after infection, in 100 μl of ice-cold lysis buffer (10 mM Tris-HCl, 0.5% Triton X-100; pH 8.0) for 30 min on ice. After centrifugation of the lysates for 15 min at 13,000 × *g* and 4°C, the supernatants were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and the DNA was ethanol precipitated in the presence of 300 mM sodium acetate at −70°C. The DNA was resuspended in 10 μl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and treated with RNase A (1.0 μg/μl) for 1 h at 37°C. Aliquots of 3 μl were electrophoresed in 2% agarose gels in TAE buffer (400 mM Tris, 200 mM sodium acetate, 20 mM EDTA; pH 8.0) at 4°C and stained with ethidium bromide. *Hin*dIII-cut lambda DNA and a 50- to 1,000-bp DNA ladder were used as size markers.

DNA from infected EPC cell cultures showed a 200-bp

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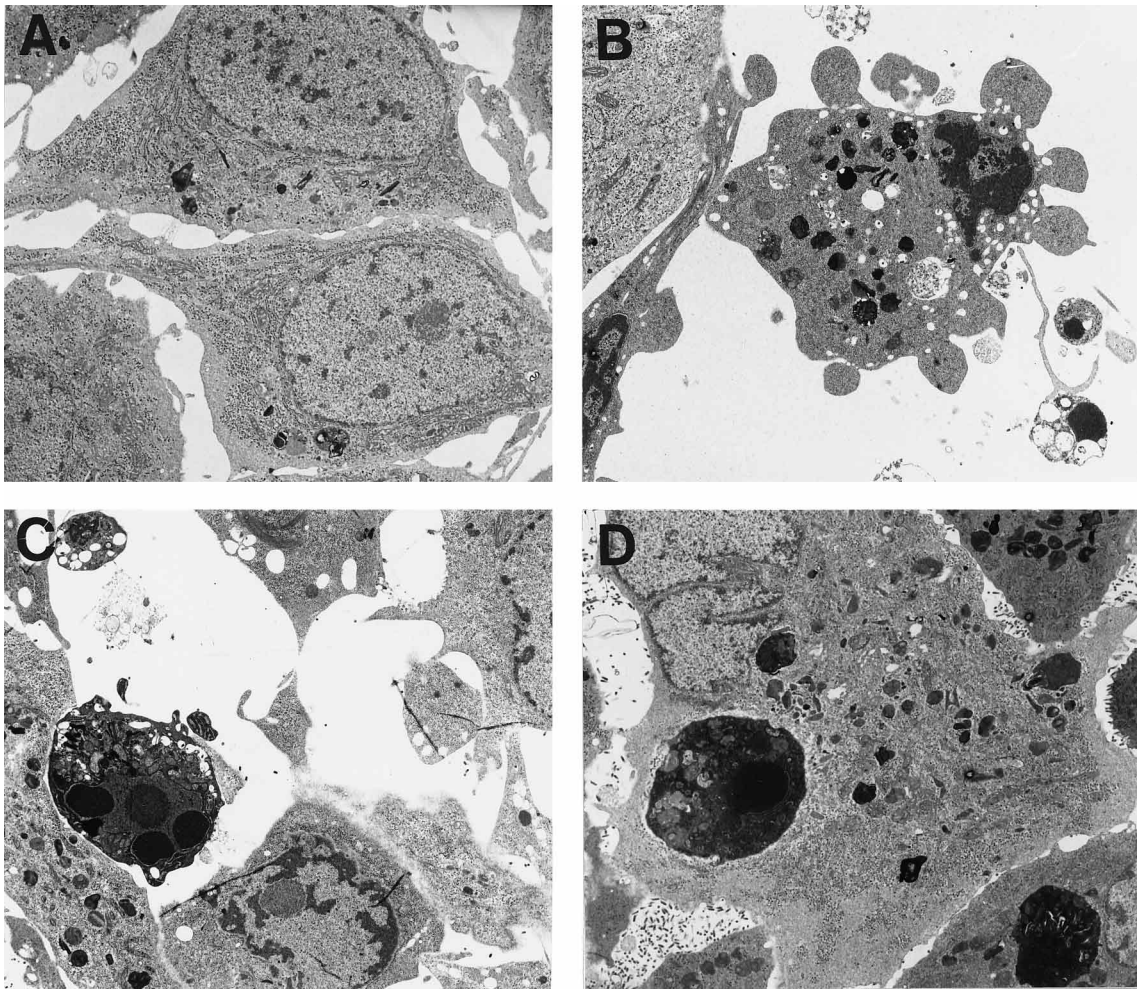



FIG. 1. Ultrastructure of mock-infected control EPC cells (A) and of SVCV-infected EPC cells that were fixed and embedded for electron microscopy at 36 h (B), 48 h (C), and 72 h (D) after infection. The infected cells show blebbing, cell shrinkage, vacuolization of the cytoplasm, condensation and breakdown of the nuclei, and, later, phagocytosis. These changes are characteristic of apoptosis. Magnification, ×3,000.

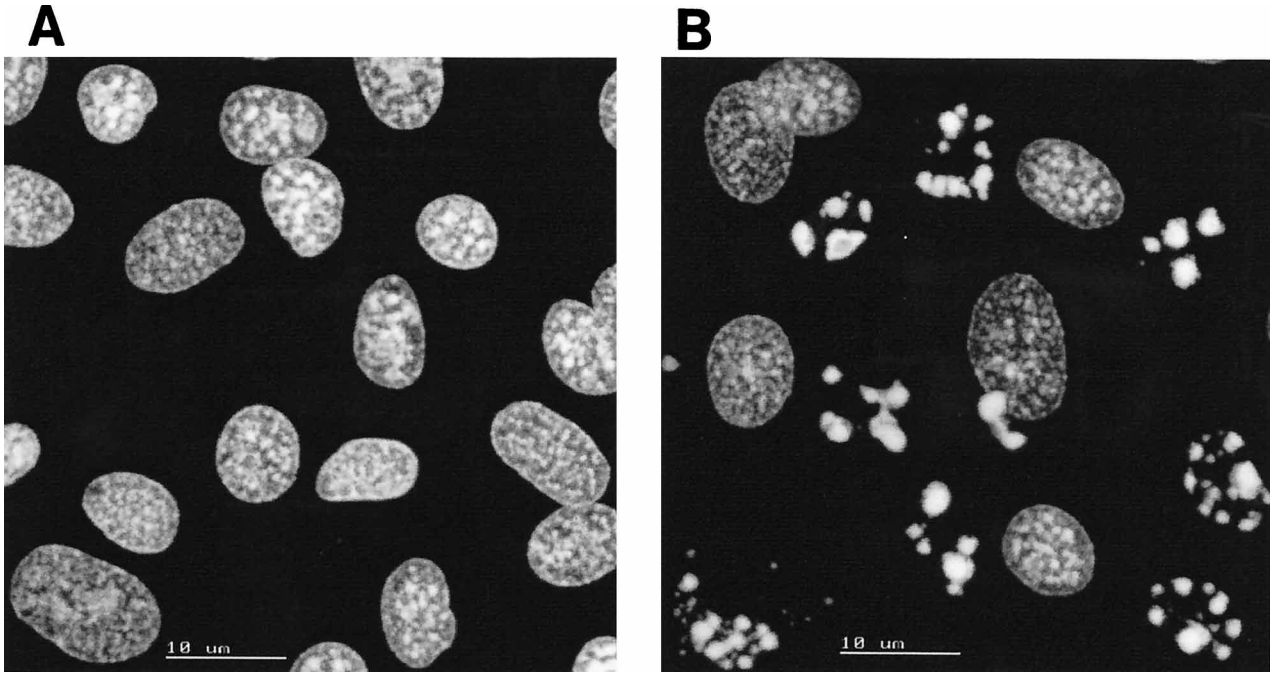



FIG. 2. Morphology of cell nuclei in mock-infected EPC cells (A) and in EPC cells infected with SVCV (B). The cells were fixed in ethanol and stained with propidium iodide 72 h after virus infection. Virus-infected cells show breakdown and condensation of nuclei. Bars, 10 μm.

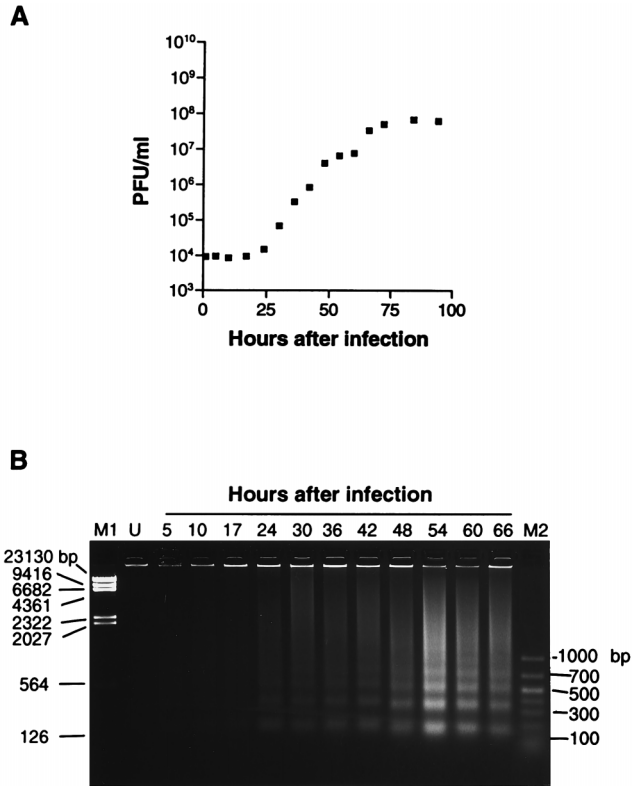



FIG. 3. Virus growth (A) and time course of DNA fragmentation (B) in EPC cells infected with SVCV. For determination of virus growth, EPC cells were grown in 24-well plates ($10^5$ cells per well) and infected with SVCV at a multiplicity of infection of 0.1. At time points ranging from 5 to 96 h after infection, cell culture supernatants were removed for virus titer determination. The virus titers were assessed by plaque assay and presented as PFU per milliliter (A). After the removal of the culture medium for virus titer determination, the cells from the respective wells were lysed for DNA fragmentation analysis (B) as described in the text. Lanes: M1, lambda-*Hin*dIII size markers; U, DNA from uninfected EPC cells; M2, 50- to 1,000-bp size markers. Lanes marked 5 to 66 contain DNA from SVCV-infected EPC cells extracted 5 to 66 h after virus infection. The virus titer starts to increase at 24 h after infection. At the same time, the cells begin showing a characteristic DNA fragmentation pattern of apoptosis, which becomes more intense as the virus titer increases.

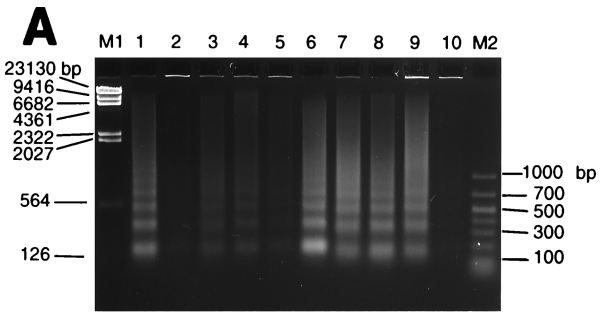
ladder pattern indicative of oligonucleosomal DNA fragmentation. The DNA fragmentation became evident simultaneously with the rise in the virus titer (at 24 h after infection), indicating that active SVCV replication and production of progeny virus is necessary to induce apoptosis in EPC cells (Fig. 3).

Taken together, the results indicate that EPC cells infected with SVCV undergo apoptosis. Infection with two other fish-pathogenic rhabdoviruses, infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus, induced similar morphological changes in EPC cells, including fragmentation of DNA into a ladder pattern (results not shown), suggesting that apoptosis may be a general cell killing mechanism of rhabdoviruses.

To further characterize SVCV-induced apoptosis in EPC cells, we examined the effect of zinc, which is known to inhibit apoptosis-related DNA fragmentation in some cell systems (6, 28). $ZnSO_4$ and $ZnCl_2$, added to the cell culture medium at concentrations ranging from 100 to 500 μM 0, 5, 24, and 48 h after infection with the virus, did not inhibit CPE or DNA fragmentation in SVCV-infected EPC cells (results not shown).

Proteolysis, through activation of interleukin-1β-converting enzyme-like cysteine proteinases, has been inferred to be involved in the process of apoptosis, and proteinase inhibitors have been shown to prevent or delay apoptosis in some cell systems (3, 5, 8, 17, 24, 25, 30). We therefore tested the effect of some proteinase inhibitors on SVCV-induced apoptosis in EPC cells. The cysteine proteinase inhibitors E-64 and the synthetic peptides Z-Phe-Phe-$CHN_2$ and Z-Phe-Ala-$CHN_2$ (20 μM each) did not inhibit the virus-induced DNA fragmentation (Fig. 4A, lanes 6 to 8) or the morphological changes (data not shown) in the SVCV-infected EPC cells. Likewise, the serine proteinase inhibitor dichloroisocoumarin (100 μM) (Fig. 4A, lane 9) failed to inhibit the DNA fragmentation and morphological changes. Treatment of EPC cells with the human endogenous ACPI at the time of SVCV infection, however, inhibited the DNA fragmentation (Fig. 4A, lanes 3 to 5) as well as the virus-induced changes in the morphology of the cell nuclei (Fig. 4C). No cytopathic effect appeared in ACPI-treated (40 nM) SVCV-infected cells up to 96 h after virus infection, while the monolayers of the untreated cells were completely destroyed by the virus infection at this time point (data not shown). At 72 and 96 h after infection, the virus titers produced by the ACPI-treated and untreated SVCV-infected cells were not significantly different (at 72 h, $5.7 \times 10^6 \pm 1.1 \times 10^6$ and $6.5 \times 10^6 \pm 2.1 \times 10^6$ PFU/ml, respectively [$n = 4$]; at 96 h, $2.4 \times 10^7 \pm 1.4 \times 10^7$ and $2.2 \times 10^7 \pm 1.4\ 10^7$ PFU/ml, respectively [$n = 4$]), indicating that the ACPI did not inhibit virus replication in the cells at these time points (Fig. 5). Treatment of SVCV-infected cells with cycloheximide (1 μg/ml) also completely inhibited the DNA fragmentation (Fig. 4A, lane 10), but cycloheximide also inhibited virus replication in EPC cells (results not shown). The ACPI used in these studies was purified from human palatine tonsils by papain affinity chromatography and chromatofocusing (2, 13). Two separately purified batches of ACPI were used, and they gave identical results. Controls with purification buffer lacking ACPI had no inhibitory effect on apoptosis (results not shown).

ACPI is a 98-amino-acid, 11-kDa protein that belongs to the cystatin family of endogenous cysteine proteinase inhibitors (1, 4, 21). In contrast to E-64 and the synthetic peptides Z-Phe-Phe-$CHN_2$ and Z-Phe-Ala-$CHN_2$, ACPI does not form covalent bonds with cysteine proteinases but instead covers the active-site cleft of the enzyme, blocking access to the active site (4). ACPI is known to inhibit mammalian cysteine proteinases such as cathepsins B, H, L, and S but not serine proteinases such as trypsin and chymotrypsin (2). In humans, ACPI is present in squamous epithelial cells, in antigen-presenting dendritic reticulum cells of lymphoid tissues (22, 26), in neutrophils (7), and in reserve cells of the prostate gland (27). Although ACPI is the oldest known endogenous inhibitor of cysteine proteinases in mammals (14) and is one of the major soluble proteins of squamous epithelial cells, the physiological functions of ACPI still remain largely unknown (4, 21). The expression of ACPI has, however, been shown to be elevated in epidermal squamous cells of persons with proliferative disorders such as psoriasis (12). Alterations in the balance between endogenous cysteine proteinase inhibitors and cysteine proteinases have also been postulated to contribute to malignant progression (4). Recently, ACPI-like papain-inhibiting cysteine proteinase inhibitors were identified in



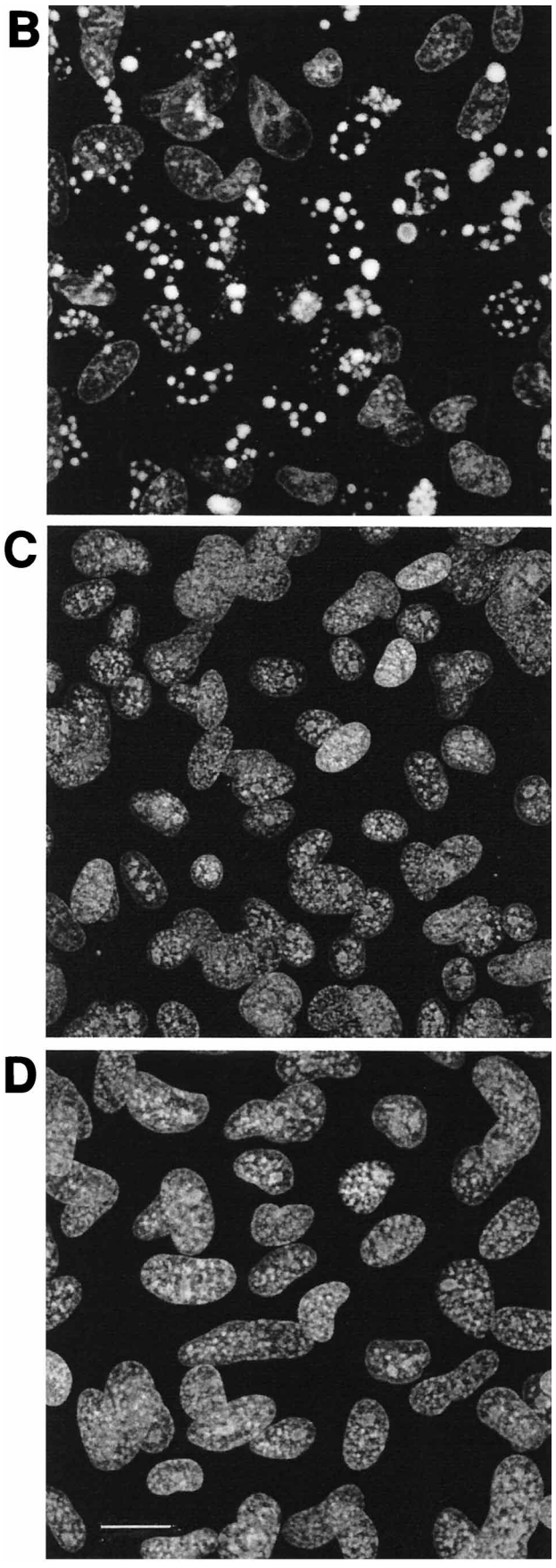




FIG. 4. The effect of proteinase inhibitors on SVCV-induced apoptosis in EPC cells. (A) DNA fragmentation assay at 72 h after virus infection. Lanes: M1, lambda-*Hin*dIII DNA size markers; 1, SVCV-infected cells; 2, uninfected cells; lanes 3 to 5, SVCV-infected cells treated with ACPI at 5, 20, and 40 nM, respectively, at the time of virus infection; 6, SVCV-infected cells treated with 20 μM E-64; 7, SVCV-infected cells treated with 20 μM Z-Phe-Phe-$CHN_2$; 8, SVCV-infected cells treated with 20 μM Z-Phe-Ala-$CHN_2$; 9, SVCV-infected cells treated with 100 μM dichloroisocoumarin; 10, SVCV-infected cells treated with 1 μg of cycloheximide per ml; M2, 50- to 1,000-bp size markers. (B) Confocal microscopy image of propidium iodide-stained nuclei of SVCV-infected EPC cells 72 h after infection. (C) Confocal image of SVCV-infected EPC cells, treated with 40 nM ACPI at the time of infection, 72 h after infection. (D) Mock-infected control EPC cells. ACPI inhibits DNA fragmentation as well as condensation and breakdown of nuclei in SVCV-infected cells. Bar, 10 μm.

epithelial cells of Atlantic salmon (20a), suggesting that ACPI-like cysteine proteinase inhibitors may be an evolutionarily conserved feature common to vertebrate epithelial cells.

Our results indicate that ACPI blocks or delays SVCV-induced DNA fragmentation and apoptosis-related morphological changes in EPC cells. The present work is the first report on a potential physiological function of the human endogenous ACPI as an inhibitor of apoptosis. The specific mechanisms by which ACPI interferes with SVCV-induced apoptosis in EPC cells are not known, but it is tempting to speculate that ACPI may inhibit interleukin-1β-converting enzyme-like cysteine proteinases. The endogenous cysteine proteinase inhibitors represent the final posttranslational level at which cysteine proteinase activity can be regulated in the cell and may, therefore, have important functions in cellular homeostasis and apoptosis.

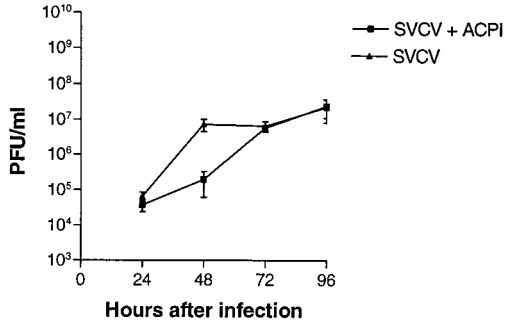



FIG. 5. Growth of SVCV in untreated and ACPI-treated EPC cells. In the ACPI treatments, 40 nM ACPI was added to the cell cultures at the time of virus infection. ACPI delayed the growth of SVCV at 48 h after infection. No inhibition of virus growth was observed at 72 and 96 h after infection. Data are means ± standard deviations ($n = 4$).

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