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Decreased messenger RNA translation in herpesvirus-infected arterial cells: Effects on cholesteryl ester hydrolase

(arteriosclerosis/cholesterol metabolism/host shutoff/mRNA degradation)

DAVID P. HAJJAR*†‡, ANDREW C. NICHOLSON†, KATHERINE A. HAJJAR§, GLORIA N. SANDO¶, AND BARBARA D. SUMMERS†

Departments of *Biochemistry and †Pathology, National Institutes of Health Specialized Centers of Research in Thrombosis, and §Department of Pediatrics, Cornell University Medical College, New York, NY 10021; and ¶Department of Medicine, National Institutes of Health Specialized Center of Research in Atherosclerosis, Cardiovascular Center, The University of Iowa, College of Medicine, Iowa City, IA 52242

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ABSTRACT **Herpes simplex viruses (HSVs) contain a function that can cause the degradation of host mRNA and mediate the shutoff of host protein synthesis. Previously, we observed that HSV infection causes a 40-fold increase in cholesteryl ester (CE) accretion in arterial smooth muscle cells due, in part, to a substantial decrease in CE hydrolysis. In studies reported herein, we found that HSV infection leads to reduced immunoprecipitable lysosomal (acid) CE hydrolase (ACEH) and β-galactosidase, another lysosomal enzyme in vascular smooth muscle cells. The HSV-induced reduction was greater with respect to ACEH than β-galactosidase. To determine whether degradation of host cellular mRNA or inhibition of cellular translation was responsible for decreased CE hydrolysis in HSV-infected smooth muscle cells, we utilized an *in vitro* translation system that permitted us to compensate for any mRNA degradation during viral infection. Reduced ACEH activity was observed in the total cellular RNA translation products of HSV-infected smooth muscle cells compared to uninfected cells owing to posttranscriptional modification. We conclude that the decrease in CE hydrolysis in HSV-infected smooth muscle cells is caused primarily by decreased ACEH synthesis and activity, which can contribute to CE accretion in these vascular cells.**

Viruses have been implicated as causative or accelerating factors in the pathogenesis of human cardiovascular disease (1, 2). Several experimental studies have clearly established that infection of normocholesterolemic chickens with Marek disease herpesvirus leads to chronic atherosclerosis resembling the human arteriopathy by altering arterial cholesterol metabolism and predisposing to free and esterified cholesterol accretion (2, 3). Similarly, it has been shown *in vitro* that herpes simplex virus (HSV) will alter cholesterol metabolism in human arterial smooth muscle cells (SMCs) at a low multiplicity of infection (moi) to produce significant cholesteryl ester (CE) accumulation (4). This is due to decreased CE hydrolysis and not increased CE synthesis or altered lipoprotein metabolism within the cell (4, 5). Concomitant with this metabolic effect is an inability to up-regulate CE hydrolase in herpesvirus-infected SMCs (6).

Other studies have also shown that herpesviruses are present in atherosclerotic tissue. The presence of herpesviral mRNA in human arteries (7), cytomegalovirus (CMV) antigen in cells derived from atherosclerotic tissue (8), and virions of the Herpesviridae family have been reported in human arterial tissue and atheromatous lesions (9). More recently, others have reported nucleic acid sequences of CMV in cells from human arterial tissue (10) and HSV DNA and/or HSV antigen in coronary arteries (11).

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Infection of permissive cells with HSV results in inhibition of host cell protein synthesis (12). A variety of mechanisms, including posttranscriptional modification, have been identified in virally infected cells that account for this decrease in cellular proteins. They can include (*i*) dissociation of host cell mRNA from polysomes (13), (*ii*) degradation of host mRNA (12, 14), (*iii*) competition between viral and cellular RNA for ribosomes and translation factors (13), and (*iv*) inhibition of cellular translation (15, 16). Herpesvirus infection can cause the degradation of mRNA in several cell types (17) with the exception of histone mRNA, which is not polyadenylylated mRNA (18). For example, HSV causes the degradation of actin and glyceraldehyde-3-phosphate dehydrogenase mRNA in baby hamster kidney cells (16), the latter protein being involved in the synthesis of neutral lipids and in bioenergetic metabolism (19).

Because significant CE accumulation occurs in arteries during herpesvirus-induced arteriosclerosis, we tested the hypothesis that increased CE accretion in the HSV-infected SMCs was due to reduced lysosomal (acid) CE hydrolase and less acid CE hydrolase (ACEH) activity in the RNA translation products in the HSV-infected cell and that the lack of this enzyme may have resulted from HSV-induced mRNA degradation and/or alterations in RNA translation.

MATERIALS AND METHODS

Cells and Viruses. Bovine thoracic aortic SMCs were isolated and propagated *in vitro* by methods described elsewhere (4). Only SMCs in early passage (passage 3 to passage 6) were used routinely. HSV type 1 (HSV-1) and wild-type adenovirus type 2 (Ad2) were purchased from the American Type Culture Collection. Ad2 was used as a control DNA virus to test for specificity of any HSV effect (4). The titers of herpesvirus and Ad stocks were determined by plaque assay and averaged 10^6 virus particles (tissue culture 50% infective dose) per ml of medium.

Virus Infection. Monolayers of arterial SMCs were initially infected with HSV-1 or Ad2 at a moi of ≈0.05–0.10 (4). Prior to inoculation with the viruses, SMCs were initially grown to confluency in the presence of minimum essential medium (MEM) containing 10% fetal calf serum (FCS) and 1% Fungizone, glutamine, and penicillin/streptomycin. At the time of inoculation, cells were exposed for 2 hr to the virus at a concentration to yield the necessary moi. Cells were washed twice at room temperature in phosphate-buffered

Abbreviations: CE, cholesteryl ester; ACEH, acid CE hydrolase; Ad, adenovirus; FCS, fetal calf serum; HSV, herpes simplex virus; mAb, monoclonal antibody; moi, multiplicity of infection; SMC, smooth muscle cell.

‡To whom reprint requests should be addressed at: Department of Pathology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

saline (PBS) and then refed fresh medium. Usually, cells were harvested 24 hr following virus inoculation depending on the experimental protocol described below.

Immunoprecipitation of ACEH and β-Galactosidase. Immunoprecipitation procedures were carried out according to the general methods outlined by Platt *et al.* (20). For these experiments, cells were grown to confluence in six-well Linbro plates in culture medium described earlier. Several wells were infected with either HSV-1 or Ad2 at a moi of 0.1. The remaining wells were mock-infected. The cells were inoculated for 2 hr and then washed in PBS. All treatment groups were washed again in PBS, and the cells were fed MEM for 1 hr, which was supplemented with 2% FCS, 1% glutamine, 1% Fungizone, and 1% penicillin/streptomycin minus methionine. After 1 hr, 4 μl of 40 μCi of [^{35}S]methionine (1 Ci = 37 GBq; specific activity, 1100 Ci/mmol) was added to each well for 24 hr (21). The next day, all wells were washed with PBS, scraped, and centrifuged, and the pellets were solubilized in 50 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol, 2 mM sodium deoxycholate, 2 mM EDTA, and 0.01% SDS. Lysates were then frozen, thawed, and sonicated. Rabbit anti-mouse immunoglobulin Immunobeads were used (Bio-Rad) for precipitation. To each of four Eppendorf tubes, we added 120 μl of a 1:1 (vol/vol) bead suspension. The beads were then spun down and 130 μl of cold lysate was added to each tube for 2 hr. Beads were then spun down again and washed two times in immunoprecipitation buffer.

To the uninfected or infected cell lysates, we added either nonimmune mouse IgG (10 mg/ml) or 1 mg of murine anti-ACEH monoclonal antibody (mAb) per ml, which was prepared in our laboratory, or rabbit β-galactosidase polyclonal antibody (Chemicon). Antibodies were added to lysates and they were incubated overnight, after which time we added washed Immunobeads. Tubes containing the mixture were rocked overnight at 4°C and then centrifuged at 12,000 × *g*. Beads were washed three times in immunoprecipitation buffer and then resuspended in 40 μl of 5× concentrated SDS/PAGE sample buffer with 4 μl of dithiothreitol (300 mg/ml). Pelleted Immunobeads were dispersed, boiled for 5 min, cooled, and centrifuged. Supernatants were subsequently electrophoresed in a 9% SDS/PAGE gel, and autoradiography was used to visualize the proteins (21).

Immunoprecipitates of ACEH and β-galactosidase were compared to actin protein in the uninfected and HSV-infected groups to assess the relative effects of the HSV infection on the various lysosomal enzymes. Scanning laser densitometry was used to quantify the blots.

Isolation of RNA for *In Vitro* Translation and Steady-State Levels of mRNA for the Actin Gene. Cells were harvested in 4 M guanidinium isothiocyanate containing 2-mercaptoethanol. Total cellular RNA was extracted by the method of Chirgwin (22). Preliminary experiments in our laboratory showed that a minimum of 2–3 × 10^6 cells was necessary to derive adequate amounts of total cellular RNA for blotting. The RNA concentration was determined by spectrophotometry, and the RNA was stored until use under 70% ethanol at −70°C. Following isolation, 20 μg of total RNA was electrophoresed in denaturing agarose gels containing formaldehyde (21) and then transferred overnight to a nylon filter (Zetaprobe). Prior to hybridization, the filter was prewashed in 0.1× SSC buffer (1.5 M NaCl/0.2 M sodium citrate) and 0.5% SDS for 1 hr at 65°C (21).

To assess possible mRNA degradation in HSV-infected SMCs, we hybridized an actin cDNA insert probe (Oncor, Gaithersburg, MD) to the cellular RNA from uninfected and infected SMCs to determine steady-state levels of RNA. The amount of RNA from the HSV-1-infected cells before *in vitro* translation was standardized to the amount of β-actin mRNA found in uninfected arterial SMCs.

A 770-base-pair chicken cDNA probe for β-actin was labeled by random hexamer primer extension and hybridized with the filters at 42°C in 50% formamide, 5× concentrated SSPE buffer (2 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA), 5× concentrated Denhardt's solution (0.1%; 0.02% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone), and 100 μg of salmon sperm DNA per ml for 24 hr. The filters were then washed under high stringency conditions. The final wash was done with 0.1× SSC/0.1% SDS buffer at 65% for 1 hr. The filters were air dried and exposed to x-ray film with intensifying screens at −70°C. The amounts of RNA were quantified by scanning the film with a laser densitometer to assess the effect of HSV infection on the steady-state mRNA levels of a typical structural protein—namely, actin.

Analysis of RNA and *In Vitro* Translation. mRNA was translated at 30°C for 90 min in micrococcal nuclease-treated rabbit reticulocyte lysates (Promega) in order to eliminate endogenous mRNA translation (23). In addition, canine pancreatic microsomal membranes were added (2 equivalents/ml of translation mix) to enhance posttranslational processing of proteins (24). In some experiments RNA translations were performed in the presence of [^{35}S]methionine (specific activity, 1100 Ci/mmol).

A positive control, RNA from brome mosaic virus, was translated as well as RNA from four groups: uninfected SMCs; HSV-infected SMCs (moi = 0.05); HSV-infected SMCs (moi = 0.1); and, Ad-infected SMCs (moi = 0.1). Blanks (no RNA) were also run in the assay to calculate background translation. After translation, aliquots of the translation mix were precipitated with trichloroacetic acid (25%) containing 2% amino acids. Precipitated radioactivity was then measured by liquid scintillation spectroscopy to assess efficiency of translation.

Unlabeled translations were performed for 90 min at 30°C to assay ACEH activity using total cellular RNA (≈100 μg of RNA). We used RNA concentrations and a time point that would give maximal amounts of protein to assay ACEH activity. Prior to translation, we also compensated for HSV-induced RNA degradation by adding equal amounts of RNA in all groups by normalizing for any degraded actin mRNA in the HSV-infected group.

CE Catabolic Activity. Following the 90-min translation period, the entire 300-μl translation mix was used for the assay of lysosomal (acid) CE hydrolase (ACEH) activity (4, 5). β-Galactosidase activity, a lysosomal marker enzyme, was also measured using a standard assay (25) in separate experiments to determine specificity of the HSV effect on cholesterol metabolism (4).

ACEH activity was measured as described (26) using 13 μM cholesteryl [1-^{14}C]oleate (specific activity, 55 mCi/mmol; New England Nuclear) as substrate for the reaction. The substrate was prepared as a lecithin/digitonide/taurocholate dispersion. Reactions were run in 600 μl of isotonic sucrose buffer (250 mM sucrose/1 mM EDTA/10 mM sodium acetate). pH was adjusted to 3.0 using 1 M HCl. Substrate blanks were run under identical conditions with translation buffer containing no RNA. Under these assay conditions, enzymic activity was linearly proportional to substrate and enzyme concentration as well as time of reaction.

The reactions were terminated after 75 min, and the unhydrolyzed substrate was removed by addition of methanol/chloroform/heptane, 1.4:1.3:1.0 (vol/vol), followed by the addition of 50 mM borate buffer (pH 10) (26). The mixture was agitated for 5 min and then centrifuged for 10 min at 2500 rpm to separate the phases. The amount of [1-^{14}C]oleate in the aqueous phase was determined by liquid scintillation spectroscopy. Activity was expressed as pmol of CE hydrolyzed per hr/mg of RNA used in the translation.

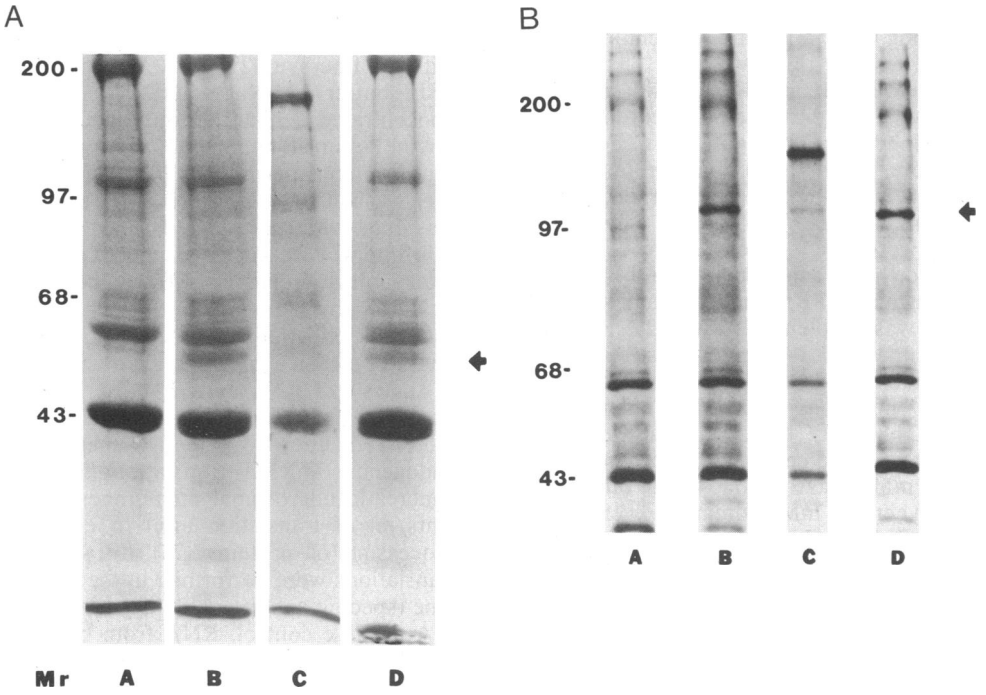

FIG. 1. (*A*) Immunoprecipitation of ACEH from a 24-hr mock-infected and HSV-infected (moi = 0.1) SMC culture. Biosynthetic labeling of cell protein was done with [^{35}S]methionine (40 μCi per 5×10^5 cells). Each lane contained ≈1 mg of cell protein or a homogenate consisting of 2×10^6 cells. Immunoisolates were subjected to SDS/PAGE, and autoradiography was performed. Lane A, a control, immunoisolate was obtained with the use of normal mouse IgG. This lane shows an absence of radioactivity at M_r 55,000 (arrow) (molecular weights shown as $M_r \times 10^{-3}$), which approximates the molecular weight of ACEH as determined under specified conditions (27). Due to the large quantity of protein loaded on these gels, several of the structural proteins such as actin (M_r 43,000), tubulin and intermediate filaments (M_r 52,000–65,000), and myosin (M_r 200,000) precipitated nonspecifically. Lane B, immunoprecipitated material isolated from uninfected, control SMCs using anti-ACEH mAb. A band is shown by the arrow corresponding to M_r 55,000. We observed that this mAb had a low affinity for ACEH consistent with its high specificity for the human enzyme against which it was prepared. Lane C, immunoprecipitated material corresponding to M_r 55,000 from HSV-infected SMCs is considerably less than that found in lane B. The levels of actin and myosin shown in lane B (and lane D) were higher than the amounts shown in lane C. We also observed a prominent protein band appearing at M_r ≈150,000 in lane C only, which may be of herpesviral origin. Lane D, immunoprecipitated ACEH from Ad2-infected cells (virus control group). Finally, we assayed ACEH activity following immunoprecipitation in another experiment to confirm that we indeed immunoprecipitated ACEH. We found that we could immunoprecipitate ≈60% of the ACEH in the cell based on our activity found in the supernatant and pellet. Control antibody (>Fc): 0 pmol of CE hydrolyzed per hr/mg of protein; ACEH antibody: 23.1 pmol of CE hydrolyzed per hr/mg of protein. (*B*) Immunoprecipitation of β-galactosidase under conditions similar to those described in *A*. Lane A, control (no antibody); lane B, uninfected SMC group; lane C, HSV-infected SMC group; lane D, Ad-infected group. In lane C, note the reduced β-galactosidase compared to lane B or D at M_r 105,000 (arrow), less actin (M_r 43,000), less myosin (M_r 200,000), and the presence of a protein possibly of herpesviral origin, at M_r ≈150,000. This protein also appeared in *A*, lane C.

RESULTS

Herpesvirus infection of arterial SMCs alters the production of ACEH, a major lysosomal enzyme (Fig. 1*A*). Immunoprecipitation of ACEH with a ACEH mAb reveals less immunoreactive ACEH in the HSV-infected SMCs (lane C) than in the uninfected cells (lane B) or the Ad2-infected SMCs (lane D). Herpesvirus infection caused a 87% decrease in ACEH protein, whereas β-galactosidase protein, another lysosomal enzyme, was decreased by 54% compared to uninfected cells (Fig. 1 *A* and *B*). Furthermore, herpesvirus infection also induced a decrease in steady-state RNA levels for β-actin mRNA (Fig. 2), an abundant protein in these cells.

In addition, when we translated mRNA from these SMC cultures to more clearly focus on the level at which HSV may affect ACEH, we found that the HSV-infected SMCs contained less ACEH activity in the RNA translation products compared to the uninfected or Ad-infected SMC groups (Table 1). Following *in vitro* RNA translation, we observed a 65% decrease in ACEH activity and a 34% decrease in β-galactosidase activity in the HSV-infected group as compared to the control groups following normalization for any degraded RNA (Table 1). Moreover, reduced efficiency of total RNA translation was also observed in HSV-infected arterial cells (Table 1) as compared to mock-infected or Ad-infected arterial cells.

DISCUSSION

We report herein that HSV infection leads to reduced levels of immunoprecipitable lysosomal CE hydrolase (ACEH) and β-galactosidase (Fig. 1), both lysosomal enzymes in arterial

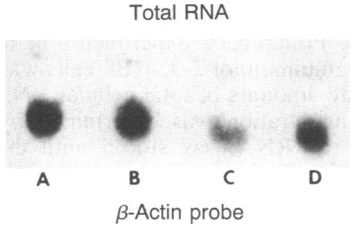

FIG. 2. Steady-state levels of β-actin gene transcripts after HSV and Ad2 infection of arterial SMCs. RNA was harvested from arterial SMCs following HSV or Ad2 infection for 24 hr. Equal quantities of RNA were electrophoresed in a 1% formaldehyde/agarose gel, blotted onto a nylon filter, and hybridized to a ^{32}P-labeled insert cDNA probe for actin. Relative amounts of actin mRNA were assessed by densitometric scanning of an autoradiogram made from the blot in order to compensate for any mRNA degraded by the HSV infection prior to *in vitro* translation (see Results, Table 1). Blot A, mock-infected; blot B, Ad2-infected, moi = 0.1; blot C, HSV-1-infected, moi = 0.1; blot D, HSV-1-infected, moi = 0.05.

Table 1. Lysosomal CE hydrolase and β-galactosidase activities in RNA translated products

SMCs	CE hydrolase activity,* pmol/hr per mg of RNA	β-Galactosidase activity,* pmol/hr per mg of RNA	% efficiency
Mock-infected	14.7 ± 1.7[a]	62.3 ± 4.9[c]	3.5[d]
HSV-infected	5.1 ± 3.1[ab]	41.1 ± 5.7[c]	2.6[d]
Ad-infected	13.0 ± 3.7[b]	57.9 ± 14.8	3.3

Data are representative of three separate experiments, each done in duplicate. Percent efficiency was calculated from the theoretical amount of translated protein produced in our *in vitro* system using brome mosaic virus as a positive control (5.9%). Infection of SMCs at a moi of 0.1 was carried out for 24 hr using either Ad2 or HSV. Values with the same superscript letter are significantly different ($P < 0.05$). *Mean ± SD.

SMCs. Reduced ACEH activity was also observed in the total cellular RNA translation products of the HSV-infected SMCs as compared to uninfected cells (Table 1). These findings support the hypothesis that reduced amounts of ACEH and its activity in the HSV-infected cell due, in part, to a lower efficiency of cellular RNA translation in these cells may contribute to significant CE accumulation in these infected cells. This decrease in efficiency may be attributed to (*i*) degradation of host cell mRNA (17), (*ii*) inhibition of host mRNA translation by virus-encoded factors (28), or (*iii*) production of factors that specifically inhibit cellular translation but facilitate viral RNA translation (28).

Herpesvirus infection also caused a decrease in steady-state RNA levels of an abundant structural protein, as shown by the decrease in hybridization signal for β-actin mRNA (Fig. 2). The decrease in mRNA encoding ACEH is likely a major factor in the substantial decrease of ACEH protein within the cell but this hypothesis could not be tested directly owing to the lack of a cDNA probe specific for ACEH. However, the HSV-induced suppression of steady-state RNA levels, as measured by mRNA transcripts for actin, cannot account solely for the 87% decrease in ACEH protein particularly since steady-state mRNA levels for actin were only reduced by 48%. This, of course, assumes similar RNA stability of these two messages following HSV infection. Thus, these findings provide further support that HSV infection may be semiselective for certain proteins in these mesenchymal cells. Interestingly, Ad, another DNA virus, did not cause RNA degradation or affect immunoreactive levels of actin, ACEH, or β-galactosidase in these arterial cells. Since we observed decreased translation of the mRNA from HSV-infected SMCs and less ACEH activity in the RNA translated products (Table 1), it is probable that the 65% reduction in ACEH activity is due not only to HSV-induced mRNA degradation but also a decrease in the translational efficiency for the remaining mRNA for ACEH. This hypothesis is supported by our observation that the percent efficiency of mRNA translation in the HSV-infected arterial SMCs is reduced by 30% even after compensation for any degraded mRNA. Viral-encoded proteins or other altered cellular factors could conceivably inhibit additional lysosomal metabolic function during infection (28).

Chemical, immunologic, and viral injuries to arteries have been proposed as initiating events in the genesis of human atherosclerosis, but the specific events involved that culminate in occlusive CE-rich arterial lesions are unknown. Herpesvirus DNA, RNA, and viral antigens have been demonstrated in human atherosclerotic vascular tissue, but a cause-and-effect relationship has not been established. However, such a relationship has been established in animal models linking viral infection and vascular pathologic changes resembling the human arteriopathy. We have demonstrated that infection of normocholesterolemic chickens with Marek disease herpesvirus results in proliferative vascular disease (2, 3). The "hallmark" lesion in this animal model (as in human atherosclerosis) is the marked accumulation of CE in arterial SMCs. We have shown that this arterial CE accretion in this avian model results from reduced CE hydrolysis owing to an inability to activate arterial CE hydrolase (3, 5, 6). We have extended these studies to human arterial tissue and observed similar effects in HSV-infected bovine and human SMCs *in vitro* (4). In an another viral system, it has also been shown that fibroblast-like cells transformed by a simian virus 40 DNA virus can accumulate 5–10 times the amount of CE compared to the parent cell line (29).

In summary, it has been proposed that herpesviruses may play a role as an etiologic or early aggravating agent in exacerbating atheroarteriosclerosis (2–4, 30, 31). The reduction of hydrolytic activities in the arterial cell induced by HSV infection suggests a posttranscriptional modification such that translation of the message transcripts for ACEH, in particular, may be altered, leading to decreased ACEH levels and its activity. This reduction in CE catabolism can predispose to CE accumulation in the HSV-infected arterial cell.

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