# Herpes Simplex Virus Infection in Human Arterial Cells

## Implications in Arteriosclerosis

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## Abstract

**Herpesviruses have been implicated as etiologic factors in the pathogenesis of human arteriosclerosis. We have examined the pathobiological effects of human herpes simplex virus (HSV-1) infection in influencing lipid accumulation and metabolism in human and bovine arterial smooth muscle cells (SMC). Significantly greater amounts of saturated cholesteryl esters (CE) and triacylglycerols (TG) accumulate in HSV-1–infected human and bovine arterial SMC than uninfected cells. This CE accumulation results, in part, from decreased CE hydrolysis. Furthermore, arachidonate-stimulated, HSV-1–infected arterial SMC have a reduced capacity to produce prostacyclin (an agonist of intracellular CE hydrolytic activity) than uninfected, stimulated SMC. It appears that HSV-1 may induce lipid accumulation in arterial SMC similar, in part, to the lipid accumulation observed in vivo during human atherogenesis. Thus, herpesviruses may contribute to lipid accumulation, which is a characteristic feature of atherosclerosis.**

## Introduction

Advances in recent clinical and epidemiological research have shown that the etiology of human arteriosclerosis is multifactorial and remains undefined. Risk factors, such as hypercholesterolemia, smoking, hypertension, diabetes, and behavioral patterns may initiate and exacerbate cardiovascular disease (1). Undoubtedly, both genetic and environmental factors contribute to the predisposition for the arteriopathy in ways not yet understood. However, inasmuch as identified risk factors are associated with the increased incidence of arteriosclerosis in Western populations, epidemiological studies suggest that these factors do not fully explain the extent and severity of the disease in the human population (2). Unidentified factors must then contribute to the etiology and pathogenesis of the disease.

Several recent studies have suggested that herpesviruses, which are ubiquitous, may be among these unknown etiologic factors. Firstly, Benditt et al. (3) found herpesviral messenger RNA in arterial plaque cells of patients undergoing coronary bypass surgery. These findings led to their proposal that expression of at least part of the herpesviral genome in the arterial wall may contribute to the initiation of intimal hyperplasia, a characteristic feature of human atherogenesis.. Secondly, Gyorkey et al. (4) have also confirmed the presence of herpesviridae antigens in cells from human atheromatous lesions. Thirdly, human arterial smooth muscle cells (SMC)[1] can be infected with herpesviruses, and herpesviruses such as cytomegalovirus replicate in these cells (3, 5). Fourthly, chronic atherosclerosis is reproducibly induced in specific pathogen-free chickens by infection with Marek's disease herpesvirus (MDV) (6, 7). In this animal model, fibro-proliferative arterial lesions were observed which contained significant quantities of cholesterol (CHOL) and cholesteryl esters (CE) (7). Increased CE synthetic activity and decreased CE hydrolytic activities were also observed in these herpesvirus-infected arteries (7). Similar alterations were observed in the lipid profile and metabolism of cultured avian arterial SMC infected with MDV (8, 9). Taken together, these findings suggest that herpesviruses may alter lipid accumulation and modulate lipid metabolism in human arterial SMC similarly to the known characteristic features of the arterial wall, during human arteriosclerosis.

We report for the first time that herpes simplex virus (HSV-1) infection causes CE accumulation in human and bovine arterial SMC due, in part, to altered lysosomal and cytoplasmic CE hydrolysis.

## Methods

Human fetal and bovine adult arterial SMC were cultured as previously described (10) and infected with 0.1–5.0 multiplicities of infection (MOI) of human herpes simplex I for various time periods. Human fetal arteries were obtained from The New York Hospital (New York, NY). Human fetal arterial SMC were selected because they are usually specific pathogen-free, unlike adult arterial SMC, which may have been previously infected with HSV-1. Under similar culture conditions, we infected replicate cell cultures with adenovirus (Ad-2), which served as a control to assess general metabolic effects after infection with another large DNA virus. Both HSV-1 and Ad-2 were gifts from Drs. Paula Traktman and Kenneth Berns of this institution. HSV-2 was also tested in preliminary studies. HSV-2 produced similar effects on CE accumulation in these cells, as did HSV-1 (data

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1. *Abbreviations used in this paper:* ACAT, acyltransferase; ACEH, acid CE hydrolase activities; Ad-2, adenovirus; CHOL, cholesterol; CE, cholesteryl ester; HSV-1, herpes simplex virus; LDH, lactate dehydrogenase; MDV, Marek's disease herpesvirus; MEM, minimal essential medium; MOI, multiples of infection; NCEH, neutral CE hydrolase activities; $PGI_2$, prostacyclin; SMC, smooth muscle cells; TC, triacylglycerols.

not shown). However, since fetal human SMC were in very short supply, we limited our studies of CHOL metabolism to SMC infected with HSV-1.

In addition to the metabolic changes after exposure to HSV-1, infection of human bovine arterial SMC was confirmed both by observation of cytopathologic effects (vacuolization with subsequent rounding of cells) and by demonstration of specific viral DNA in SMC nuclei after in vitro hybridization. Kit 855 for HSV-1 and kit 851 for Ad-2 (Enzo Biochem, Inc., New York, NY) were used for in vitro hybridization studies. Cytopathologic effects were observed in Ad-2–infected SMC but they were not as pronounced as in HSV-1–infected cells.

To measure lipid accumulation in HSV-1–infected SMC, lipid analyses were performed by extracting SMC (11), separating the lipids by TLC (12), and quantitating these lipids by gas–liquid chromatography (GLC) (13) or by scanning TLC fluorometry (12).

Lysosomal (acid) and cytoplasmic (neutral) cholesteryl esterase activities were assayed by methods previously described (9, 14). Activity of acyl coenzyme A/cholesterol acyltransferase (ACAT) was also measured by procedures outlined previously (7, 9). Production of prostacyclin ($PGI_2$), measured as 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) (the stable hydrolytic product of $PGI_2$), was measured by radioimmunoassay (15). Marker enzyme activities were assayed by the methods of Peters et al. (16).

Statistical analyses of the data were done by analyses of variance as described elsewhere (17).

## Results and Discussion

Results indicate that maximal accumulation of lipids occurred with an MOI equalling 1, using HSV-1 for 2 h followed by 3 d incubation of SMC at 37°C. Infection under these conditions caused focal detachment of the cells, widespread blebbing, and vacuolization. Cellular debris was also associated with the HSV-1–infected SMC and any exposed part of the plastic dish. Many infected cells and cellular debris were Oil red 0 positive; however, Oil red 0 staining was not always demonstrable. When a higher MOI was used, cytopathologic effects were severe, reflecting the lytic nature of the human herpes virus infection, particularly in the human SMC compared with the infected bovine SMC.

As shown in Table I, HSV-1 infection of human SMC produced a 43-fold increase in CE and more than a 7-fold increase in triacylglycerols (TG) as compared with uninfected SMC. Lipid accumulation in HSV-1–infected bovine SMC was characterized by a 19-fold increase in CE and a 2-fold increase in TG. Since extracellular debris was also present, the large increases in CE may also represent extracellular lipid. No significant lipid accumulation could be documented in Ad-2 virus-infected human or bovine SMC (data not shown).

Fatty acid analysis of CE revealed a significant increase in all major fatty acids (Table II), including palmitate (C 16:0), stearate (C 18:0), oleate (C 18:1), linoleate (C 18:2), and linolenate (C 18:3). The level of cholesteryl arachidonate (C 20:4), however, was unaltered after HSV-1 infection. The CE profile of fetal bovine serum, which is used to maintain these cells in culture, does not approximate the percent distribution of CE in either the uninfected cells (controls) or the HSV-1–infected SMC. These findings suggest that the CE from fetal bovine serum does not contribute to the intracellular pool of CE.

We also observed that the HSV-1–infected cells metabolize CE differently than uninfected cells with respect to uptake, degradation, and storage within the cell. This is particularly evident regarding the polyunsaturated/saturated fatty acid ratio of CE in HSV-1–infected cells (9:91) compared with the ratio seen in uninfected cells (51:49). The increase of cholesteryl stearate in these HSV-1–infected cells is similar to our results found in avian SMC after infection with an avian herpesvirus (8). This observation may represent a selective decrease in the rate of CE hydrolysis in the HSV-1–infected SMC. The mechanism responsible for saturated CE accumulation in these cells requires further elucidation.

Lipid accumulation in bovine SMC was similar using an MOI of 5 for HSV-1, as compared with an MOI of 1. An MOI of 5 was the highest ratio of virus to bovine arterial SMC we could use without causing rapid lysis. In human SMC, we found that an MOI of 5 resulted in rapid lysis. We speculate that the ability to use higher levels of virus with the bovine cells could be due to lower sensitivity of these cells to virus or to differences in the metabolic consequences after infection. No specific surface binding protein has yet been found for HSV-1; however, since it infects mostly humans, such a receptor is

*Table I. Lipid Accumulation in HSV-1–infected Human and Bovine Arterial SMC*

| Cells | Sample | CE | CHOL | TG |
|---|---|---|---|---|
| Human SMC | Control | 3±1*‡ | 28±1 | 8±2§ |
| | HSV-1 | 128±31‡ | 29±2 | 60±7§ |
| Bovine SMC | Control | 2±1‖ | 32±1 | 29±11¶ |
| | HSV-1 | 38±18‖ | 31±1 | 55±12¶ |

SMC (p-5) were cultured with 2 ml/well of minimal essential medium (MEM, Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) and were allowed to become almost confluent at 37°C in a flowing 5% $CO_2$–95% air incubator. Cells were washed with Hanks' balanced salt solution (HBSS) (Gibco) and cultured with fresh MEM (2% FCS) to prepare them for inoculation. For infection, MEM (2% FCS) containing $10^6$ plaque-forming units/ml of HSV-1 was added per well (MOI = 1). Final volume in each well was 2.3 ml. Cells were incubated for 2 h, after which medium was removed by aspiration, cells were washed three times with 2 ml of HBSS, and then were cultured with fresh MEM (20% FCS). Incubation was continued for an additional 48–72 h at 37°C. Medium was then removed and cells were washed with HBSS. Total lipids were extracted (11). Extraction was repeated twice. Extracts were pooled, evaporated under $N_2$ (gas), and the residue was taken up in $CHCl_3$. The cell monolayer was then overlaid with 2 ml of 0.2 N NaOH for 2 h to assay cell protein (18). After addition of beta sitosterol to the lipid extract, aliquots of the cell lipid extract were assayed for free and esterified CHOL content by GLC (13). CE was hydrolyzed by alkaline hydrolysis, followed by reextraction with hexane. CHOL was separated and quantified on a 6-ft OV-17 column (3%) on Chromosorb W 100 to 120 mesh (Supelco, Bellefonte, PA) using a thermal gradient program as follows: 260°C for 2 min, 3°C/min to a final temperature of 275°C. Mass of CE was determined by the difference of the total CHOL (alkaline hydrolysate) minus the mass of free CHOL. * X±SD; $n = 3$, separate experiments. Each analysis was done in triplicate for a given experiment with human cells, and sextuplicate for bovine cells. Units are expressed as micrograms per milligram protein. Values with the same symbols are significantly different ($P < 0.05$). CE and CHOL were assessed by GLC analyses (13); TG were quantitated by scanning TLC fluorometry (12).

*Table II. Fatty Acid Profile of CE in HSV-1-infected Human Arterial SMC*

| Fatty acid esterified to CHOL | | | | | | |
|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:4 |
| Control ($n$ = 4) | 0.17±0.04* (27) | 0.14±0.04 (22) | 0.11±0.04 (17) | 0.06±0.02 (9) | 0.12±0.02 (19) | 0.04±0.02 (6) |
| HSV-1 ($n$ = 6) | 0.31±0.10 (2) | 14.50±7.60 (89) | 0.51±0.19 (3) | 0.13±0.04 (1) | 0.76±0.16 (4) | 0.04±0.03 (1) |
| Fetal bovine serum ($n$ = 2) | (28) | (11) | (39) | (11) | (Trace) | (11) |

Except for C 20:4, all values between controls and HSV-1-infected human arterial SMC were significantly different ($P < 0.05$) for each CE. The fatty acid profile of cellular CE was determined by GLC after thin-layer chromatographic separation of CE from other cellular lipids and subsequent conversion to their corresponding methyl esters using boron trifluoride/methanol and *sn*-1,2,diheineicosanoylphosphatidylcholine as an internal standard (13). Fatty acid methyl esters were separated on a 6-ft SP-2340 column using a thermal gradient as follows: 160°C for 5 min, 5°C/min to a final temperature of 215°C. * X±SD. Units are expressed as micrograms per milligram protein. Values in parentheses represent the percent distribution of CE fatty acids.

likely to be in lower number on the surface of these bovine SMC.

When cultured cells undergo minimal or severe injury either by mechanical, chemical or viral means, they release cytoplasmic lactate dehydrogenase (LDH) into the postculture fluid. To determine if HSV-1 infection alters CE metabolism as a result of generalized cell injury, the release of LDH was measured in the postculture fluid after both HSV-1 and AD-2 infection (19). Neither bovine nor human SMC released detectable amounts of LDH after AD-2 infection; however, there was a 5–12% increase in the release of LDH activity above uninfected cultures after HSV-1 infection. Thus, it is likely that the lipid accumulation (CE accretion) observed after HSV-1 infection may result from either cell injury or it is a specific HSV-1-induced metabolic effect.

To evaluate a mechanism responsible for herpesvirus-induced CE accumulation in human and bovine fetal arterial SMC, we studied the CE cycle in both infected and uninfected cells (9). To assess CE hydrolysis, we measured lysosomal (acid) and cytoplasmic (neutral) CE hydrolase activities (ACEH, NCEH) (E.C. 3.1.1.13) (9) (Table III). ACEH activity was decreased by 35% ($P < 0.05$) in HSV-1-infected SMC (both human and bovine). Furthermore, we observed a 57 and 84% decrease of cytoplasmic NCEH activity in infected human and bovine arterial SMC, respectively. No significant alteration in ACAT activity was demonstrated in either cell type after HSV-1 infection. No significant decrease in ACEH or NCEH could be demonstrated after Ad-2 infection (data not shown). These data suggest that the accretion of CE in HSV-1-infected cells results, in part, from decreased CE catabolism and not from altered anabolism.

Since we previously showed that CE hydrolysis is enhanced by eicosanoid agonists of adenylate cyclase such as $PGI_2$ (15), we tested the hypothesis that the lower CE hydrolytic activity in HSV-1-infected SMC resulted from a decreased capacity of HSV-1-infected SMC to produce $PGI_2$. As shown in Table IV, we observed less spontaneous production of $PGI_2$ after the cells were infected for 3 d than in uninfected SMC, as well as markedly lower production of $PGI_2$ after stimulation with sodium arachidonate for 30 min. These data support the hypothesis that the reduction in ACEH and NCEH activities in the HSV-1-infected SMC may be a consequence of lower $PGI_2$ production by these SMC.

Since ACEH is a lysosomal enzyme and NCEH is a cytoplasmic enzyme, we examined the selectivity of HSV-1 infection on organellar activity by examining the activity of two other lysosomal marker enzymes, namely *N*-acetyl-β-glucosaminidase and β-galactosidase, and one cytoplasmic marker enzyme, neutral-α-glucosidase. These assays were carried out to determine if HSV-1 has a general effect on lysosomal or cytoplasmic enzymic activities (9). The data in Table V indicate that these marker enzyme activities do not change significantly in human or bovine SMC after HSV-1 infection, suggesting that the effects of HSV-1 infection on CE metabolizing enzyme activities is specific and does not represent a general reduction in lysosomal or cytoplasmic metabolism at this low MOI.

Although human HSV-1 is more lytic for human arterial

*Table III. CE Metabolism in HSV-1-infected Human and Bovine Arterial SMC*

| | ACEH | | NCEH | | ACAT | |
|---|---|---|---|---|---|---|
| Sample | Human | Bovine | Human | Bovine | Human | Bovine |
| Control | 385±35*‡ | 566±80*§ | 58±3*‖ | 31±7*¶ | 50±31* | 55±29* |
| HSV-1 | 249±65‡ | 350±76§ | 25±5‖ | 5±3¶ | 65±28 | 67±17 |

Cells were propagated and infected as described in Table I. After 2 h of infection and 2 d incubation, medium was removed. After two washes with HBSS, each monolayer was overlaid with 1 ml of isotonic sucrose buffer (9). Cells were scraped from the wells, homogenized, and samples were assayed for CE metabolizing enzyme activities (9). * X±SD; picomoles per hour per milligram protein; $n$ = 3 for studies with human cells; $n$ = 6 for bovine cells. Analyses were done in triplicate. Values with corresponding symbols are significantly different ($P < 0.05$).

*Table IV. $PGI_2$ Production by HSV-1-infected Bovine Arterial SMC*

| | Spontaneous release | Sodium arachidonate challenge |
|---|---|---|
| Control | 852±15*‡ | 115±4§ |
| HSV-1 | 698±60‡ | 41±7§ |

Cells were propagated and infected as described in Table I. After SMC exposure to HSV-1 to allow maximum infectivity, cells were washed twice in PBS, and MEM plus 10% FCS was added to SMC. After 72 h, medium from each well (control and HSV-1-infected SMC) was collected and assayed for 6-keto $PGF_{1\alpha}$ (spontaneous release) (15). Cells were washed twice with PBS. MEM plus 20 μM C 20:4 was then added for 30 min. Cells were incubated at 37°C, after which the medium was recovered and assayed for 6-keto $PGF_{1\alpha}$ (15) (C 20:4 challenge). Cells were then treated with 0.2 N NaOH as described in the legend to Table I for analyses of cell protein (20).
* X±SE; nanograms 6-keto-$PGF_{1\alpha}$ released into medium per milligram protein. Analyses were done in sextuplicate in two separate experiments. Values reported here are for one experiment. Values with corresponding symbols are significantly different ($P < 0.05$).

SMC than MDV for avian arterial SMC, (MOI > 1), our observation that HSV-1 infection can decrease both lysosomal and cytoplasmic CE hydrolytic activities in human and bovine arterial SMC parallels our previous findings with MDV-infected avian arterial SMC (9). Possible explanations for altered CE metabolism include the following: (*a*) viral DNA produces a modified cholesteryl esterase with lesser cellular activity; (*b*) the virus produces inhibitors (for CE hydrolase) or promotors (for lipoprotein-CE uptake) in the cell; or (*c*) the presence of large endogenous pools of CE regulate their intracellular metabolism by feedback responses on hydrolysis (7).

We could not demonstrate a significant increase in CE synthetic activity in these HSV-1-infected, fetal human arterial SMC as others have found in adult human atheromatous arteries. However, many of the other in vitro findings reported herein provide supportive evidence that there may be a causal relationship between herpesvirus infection and arteriosclerosis, since this herpesvirus does cause CE accumulation (Table I). We know that arteriopathies are responsible for more deaths than any other disease in the Western world. Because (*a*) the adult population can be infected concurrently with up to five different herpesviruses (3), (*b*) arterial cells contain herpesviral antigens (20), (*c*) herpesvirus DNA is found in arterial lesions of atherosclerotic patients (4), and (*d*) human herpesviruses can cause significant lipid accumulation, these studies encourage us to further define the molecular mechanisms of herpesviral expression that could lead to alterations in CE metabolism and accumulation during arteriosclerosis.

*Table V. Lysosomal and Cytoplasmic Marker Enzyme Activities in HSV-1-infected Human and Bovine Arterial SMC*

| | Lysosomal | | | |
|---|---|---|---|---|
| | NAGase | | β-galactosidase | |
| Sample | Bovine | Human | Bovine | Human |
| Control | 3,138±1,577*‡ | 10,946±4,333 | 900±172 | 1,411±125 |
| HSV-1 | 3,773±640 | 8,924±3,626 | 872±89 | 1,616±413 |

| | Cytoplasmic | |
|---|---|---|
| | Neutral-α-glucosidase | |
| Sample | Bovine | Human |
| Control | 1.1±0.7 | 2.1±0.6 |
| HSV-1 | 1.8±0.6 | 2.7±0.5 |

Cells were propagated and infected as described in Table I. NAGase, β-galactosidase, and neutral-α-glucosidase activities were assayed as described previously (16). NAGase, *N*-acetyl-β-glucosaminidase.
* X±SD; $n = 6$ using bovine SMC; $n = 3$ using human SMC.
‡ Arbitrary fluor units per hour per milligram protein.

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