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WARNER–LAMBERT/PARKE–DAVIS AWARD LECTURE

Viral Pathogenesis of Atherosclerosis

Impact of Molecular Mimicry and Viral Genes

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Human atherogenesis is a pleiotropic process with an undefined cause. Several pathologic factors have been linked to the disease process, including arterial injury or activation of the endothelium, which may initiate proatherosclerotic events in the vessel wall. Atherosclerotic lesions are characterized, in part, by the presence of activated immune cells, abnormal cell proliferation, and altered cholesterol metabolism. These activated immunocompetent cells in plaques produce vasoactive mediators that can alter homeostasis and may promote the arteriopathy. Both molecular and structural evidence is presented that herpesviruses, by way of induction of altered gene function and cellular cholesterol metabolism, coupled with their ability to activate coagulation and a monocyte receptor on the infected endothelium, are involved in major pathogenic events associated with atherosclerosis and thrombosis. Work from the author's laboratory, as well as from other research groups, have shown that avian and human herpesviruses act specifically to induce alterations to the surface and inner layers of the blood vessel wall that may predispose to atherosclerosis and its attendant clinical complications. (Am J Pathol 1991, 139:1195–1211)

An important feature of the genesis of human atherosclerosis is its multifactorial nature. No single entity has been linked directly to its pathogenesis in either animal models of the disease or in the human arteriopathy. Evidence is accumulating that early developmental changes in specific vascular beds highly susceptible to the disease are paramount to the latter alterations of arterial metabolism that predisposes to subsequent atheromatous changes.[1–3] Undoubtedly these changes are amplified by risk factors such as hypercholesterolemia, hypertension, smoking, and diabetes.

For almost 20 years, interest in a viral cause of atherosclerosis has continued, spurred by the early work of Benditt and Benditt[4] and Fabricant et al.[5,6] This perseverance to define the viral cytopathologic mechanisms involved has certainly been enhanced by studies of an animal model of virally induced atherosclerosis under normocholesterolemic conditions,[6–9] and by the recognition of the association of this disease in patients who have undergone cardiac transplant surgery, but who also are infected with cytomegalovirus,[10,11] one of the seven herpesviruses now known to infect humans.[12]

Vascular injury purported to be involved in early stages of the arteriopathy is also a common feature of acute herpesvirus infection. Recent studies have identified herpesvirus antigens and nucleic acids in the vascular wall[13–18] after their passage through the endothelium. At the biochemical level, a variety of herpesvirus-induced pathobiologic changes have been documented within vascular cells that impact on cellular lipid (cholesterol)[9] and connective tissue (proteoglycans) metabolism.[19] In fact, the herpesvirus-induced alterations in lipid metabolism *in vitro* and *in vivo* parallel those pathologic events reminiscent of the arteriopathy found in the human disease.[9,20–23]

Herpesviruses induce an arterial lesion characteristic of leukocytoclastic vasculitis. There is granulocyte infiltration in the artery, accompanied by thrombin formation

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and fibrin deposition.[24] Hence this vector has the ability to influence not only atherosclerotic changes but also processes related to a thrombotic diathesis. Because the virus causes a fatty-proliferative lesion in animals that occasionally contains microthrombin,[7] such events support the hypothesis that surface expression of adhesion molecules and intimal hyperplasia may be initiated by a virally induced event. This may trigger cell proliferation by enhancement of growth factor or by cytokine release from vascular cells or adherent macrophages. Or it also may alter the balance between mitogen and growth suppressor synthesis in the vascular wall.

Based on this background information, both molecular and structural evidence are highlighted to support the hypothesis that herpesviruses, by altering gene function and cellular lipid metabolism, coupled with the ability to activate the coagulation system and a monocyte receptor on the endothelium, are involved in pathogenic events associated with human atherosclerosis. Information about candidate viral or transforming genes potentially related to atherogenesis also are presented.

Epidemiologic Evidence Linking Herpesvirus and Atherosclerosis

Herpesvirus infections are widespread in the general population.[25–27] They are ubiquitous viruses found in most tissues of the body, even in blood cells. By 10 years of age, more than 50% of children demonstrate antibodies to herpes simplex virus (HSV) type 1.[25] The incidence of herpes simplex virus, type 2, is more difficult to estimate, but two studies cite a prevalence of up to 22%.[26,27] In the 35- to 44-year-old age group, substantially more than 10% demonstrate antibodies to HSV type 2.[26,27] A more recent study demonstrated that the incidence of infection approaches 20% in the population aged 15 to 74 years.[27] It has now been documented that seven different herpesvirus can infect humans.[12]

Human atherosclerosis is not completely accounted for by all known risk factors. This suggests that the human risk factors may by synergistic with other, as of yet unidentified, initiating factors.[7,28] A direct seroepidemiologic link between cytomegalovirus (CMV) infection and atherosclerosis has been suggested.[29] A case-control study was performed wherein patients who underwent cardiovascular surgery were compared with a control group of subjects with similar cholesterol levels and epidemiologic factors but who were not undergoing surgery. In approximately 160 pairs of patients, the prevalence of CMV antibodies was higher in the surgical group than in the control group (90% and 74%, respectively), and a greater percentage of surgical cases than controls had high titers of CMV antibodies (57% and 26%, respectively). There was no correlation between antibody titers and blood levels of cholesterol or triglycerides. Interestingly a recent Framingham Heart Study failed to find an overall association between fever blisters or cold sores, with a 6-year incidence of coronary heart disease in patients 58 to 89 years of age.[30] Previous work in this group of patients had shown a strong correlation of a self-reported history with serologic evidence of previous herpes simplex virus type 1 (HSV-1) infection. This study does not completely rule out the possibility of a relationship between HSV and atherosclerosis, particularly because a subgroup of women with recurrent cold sores had twice the risk of developing coronary heart disease.

The strongest epidemiologic link between infection with herpesviruses and atherosclerosis in humans is in the heart transplant population. Several recent studies have demonstrated a strong correlation between CMV infection and accelerated atherosclerosis.[10,11] At Stanford University, 300 cardiac transplant patients were treated with immunosuppression and were followed prospectively for the occurrence of CMV infection and the development of atherosclerosis. Of those tested, 91 patients developed CMV infections, based on: 1) positive cultures for CMV, 2) demonstration of characteristic CMV inclusion bodies in tissue samples, or 3) a fourfold rise in gamma G immunoglobulin (IgG) CMV antibodies. After a 5-year follow-up, the rate of graft loss due to accelerated atherosclerosis was 69% in CMV-infected patients, but only 37% in the non–CMV-infected group.[10] Furthermore there was a 10-fold greater incidence of patients who died with more than 50% luminal obstruction of their coronary arteries. These individuals had CMV infections.[10] Similar results were reported from a somewhat smaller study at the University of Minnesota.[11] This study compared rates of CMV infection and atherosclerosis in 102 immunosuppressed patients who had received a cardiac transplant and survived for at least 1 year. At 2 years after transplant, 32% of the CMV-positive patients had coronary artery disease, as opposed to 10% of the CMV-negative patients. To date, these findings provide the strongest evidence in humans linking herpesvirus infection with atherosclerosis.

Pathologic Evidence Linking Herpesviruses with Atherosclerosis

After the publication in 1973 of a landmark study by Dr. Earl P. Benditt on the possible monoclonal nature of atherosclerotic lesions,[4] his laboratory was one of the first groups to document the presence of herpesvirus nucleic acids in arterial tissue removed during coronary bypass surgery. They used *in situ* hybridization techniques.[14] Approximately 10% of 160 tissue samples were positive

for herpesvirus. A second series of experiments examined tissues that contained abnormally thickened intima. Fifty percent of the samples reacted positively with an HSV-2 probe. Positive staining occurred in cells located in discrete foci, characterized by increased cellularity within or adjacent to the intima. Melnick and his colleagues[13,15,16] extended these studies, focusing primarily on CMV, which they were able to detect in cells cultured from arterial tissue derived from patients with advanced (grade III) atheroarteriosclerosis.

In 1984, Gyorkey et al[13] reported the detection of virions of the herpesvirus family in electron microscopic sections from aortae of 10 of 60 patients with atherosclerosis undergoing cardiovascular surgery. These viral particles in various stages of replication included empty nucleocapsids and virions with dense cores. They were present in smooth muscle and endothelial cells. Of 1360 grids that were examined, 35 showed evidence of viral presence. Petrie et al,[16] using *in situ* hybridization techniques, demonstrated increased detection of CMV in cultured smooth muscle cells from arterial plaques by staining for viral antigens. Most recently, Hendricks et al[17] showed that detection of CMV nucleic acids by the polymerase chain reaction (PCR) was possible in 90% of samples obtained from patients with severe atherosclerosis as compared with only 50% in patients with minimal or no atherosclerosis. The presence of the complete viral genome was shown in these samples by both dot blot DNA hybridization and PCR, using probes and primers derived from the immediate early and late genomic regions of CMV. Messenger RNA transcribed from immediate-early CMV genes but not the late genomic regions could be demonstrated by *in situ* DNA hybridization. This suggested that CMV exists in the vessel wall primarily in a *latent* state, where expression of immediate-early messenger RNA transcripts may occur without expression of messenger RNA coding for structural capsid proteins.[17] Perhaps this may lead to an alteration in factors that control growth in the vessel wall, predisposing to intimal thickening.

Finally, Yamashiroya and his colleagues[18] have performed both DNA hybridization and immunohistochemical experiments on coronary vessels of young trauma victims to identify evidence of viral antigens or nucleic acid sequences at *earlier* stages of the arteriopathy. Evidence for HSV or CMV was detected in 8 of 20 specimens from coronary arteries. The viral DNA or antigens were found in cells of the intact luminal surface as well as in focal clusters of spindle-shaped or foam cells in the intimal layer. Taken together, this study and those mentioned above support the hypothesis that these herpesviruses may *contribute* to early events in atherogenesis, because the herpesvirus can be found in the vasculature at various stages of the arteriopathy.

Clearly herpesviruses can infect arterial cells. In fact, HSV types 1 and 2 can infect both bovine and human vascular endothelial cells[31,32] and vascular smooth muscle cells.[22] Similarly human CMV also can infect such cells.[33,34] Infection of cells *in vitro* with HSV results in an inhibition of host cellular protein synthesis. This is depicted in Figure 1. This 'shutoff' of host cell protein synthesis occurs in two steps. The first stage, occurring in the first few hours after infection, 'early shut-off,' is mediated by a virion-associated protein and causes dissociation of host cell mRNAs from polysomes, rendering the mRNA nonfunctional and enhancing mRNA degradation.[35,36] Kefalides and Ziaie[37] have demonstrated that this mechanism is relevant to the mRNAs encoding extracellular matrix proteins in both human endothelial cells and human arterial smooth muscle cells[38] infected with HSV-1 *in vitro.* The delayed shut-off of host cell protein synthesis is thought to be mediated by a newly synthesized immediate early-viral gene product that subsequently causes degradation of cellular messenger RNAs.[39] Some of the viral genes responsible for the inhibition of host cell protein synthesis have been mapped and are discussed elsewhere.[40]

Biochemistry of Viral-induced Atherogenesis

The concept that herpesviruses play a major role in the cause and pathogenesis of atherosclerosis is supported by two observations: 1) The findings of Paterson and Cottral, reported in 1950,[41] in which neurolymphomatosis and coronary sclerosis induced by an infectious agent was linked to arteriosclerosis in chickens; and 2) intracellular and extracellular cholesterol accumulation was observed in cell cultures infected with feline herpesvirus.[5]

The work from this laboratory and those of our collaborators have shown that atherosclerosis in chickens induced by an avian herpesvirus infection can provide considerable experimental evidence to support the concept that herpesviruses are involved in the pathogenesis of atherosclerosis.[6–9] Pathogen-free normocholesterolemic chickens were infected with 100 plaque-forming units of an avian herpesvirus that causes Marek's disease (MDV). Uninfected pathogen-free chickens served as a control. Marek's disease virus (MDV) is a herpesvirus that causes malignant lymphomas of T-cell origin in this animal model.[42] Primarily nerve lesions and visceral tumors develop. An antigenically related herpesvirus to MDV, herpesvirus of turkeys (HVT), has been used successfully by the poultry industry as an effective vaccine against the neoplastic effects of MDV.[42] Nonpathogenic for turkeys and chickens, this naturally occurring herpes-

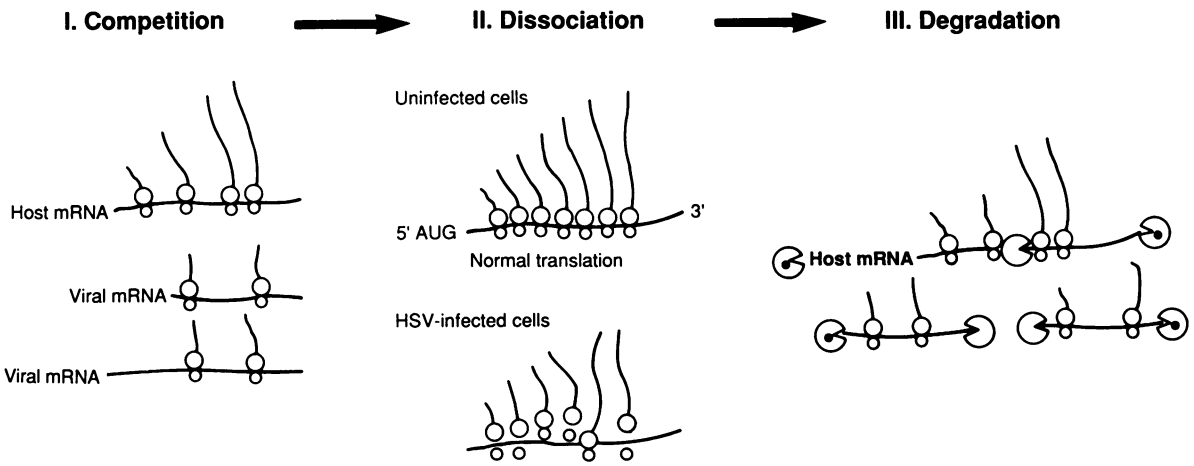

Figure 1. *Hypothetical sequence of cytopathologic changes during virally–induced atherosclerosis. This schema describes three steps that can lead to decreased protein synthesis in the mammalian cell once challenged with herpes virus. First, competition occurs where the viral mRNA can "compete" with the host mRNA in the total mRNA pool in the cell during the translation process. After several hours of viral infection, viral mRNAs can be the predominant RNA transcripts that are translated in the infected cell, producing viral proteins. In addition, dissociation of the host cell mRNA from the polysomes can occur in the infected cells, interrupting the normal translation of the RNA transcript, from the 5'3' direction on the polysome. (AUG is the initiating codon in eucaryotic cells for translation). Finally, degradation of the RNA transcripts by a virion-related or newly synthesized virally encoded factor(s) (depicted by the crescent moon figure) can occur in the host cell, predisposing to decreased RNA translation and host protein synthesis. This shut-off of protein synthesis leads to alterations in extracellular matrix and decreased production of those enzymes that are involved in lipid catabolic activity. The cytopathologic effects include cytoplasmic lipid accumulation and alterations in the extracellular matrix. Reprinted with permission.*[96]

virus elicits an immune response in chickens that protects against subsequent tumor development by MDV.[42]

A most interesting property of MDV is that it can cause atherosclerosis reproducibly in specific-pathogen-free chickens[6–9]; and that immunization of chickens with HVT can prevent MDV-induced atherosclerosis.[8,9] In fact, the distributional, morphologic, and biochemical nature of the atherosclerotic lesions induced by MDV infection closely resembled human atherosclerosis.[7] Coronary arteries, the aorta, and its branches were affected, resulting in fatty or fatty-proliferative-type lesions.[7]

We showed that aortic tissue from the normocholesterolemic, MDV-infected group had a significantly higher content of free and esterified cholesterol, triacylglycerols, and phospholipids than did uninfected controls.[9] Feeding the infected animal a high-cholesterol diet produced a synergistic effect on lipid accretion. Marek's disease virus infection also caused an abnormal rise in cholesteryl ester (CE) synthesis, with a concomitant reduction in CE hydrolysis *in vivo,* thus causing arterial lipid accumulation.[9] Infection of cells with herpesviruses *in vitro* also produced profound effects on cellular cholesterol metabolism,[20–22] which paralleled our *in vivo* data. Infection of avian smooth muscle cells with MDV *in vitro* greatly increased the accumulation of cholesterol and CE.[20] This specific type of lipid accumulation, which also occurs during the human arteriopathy, was due to decreased lysosomal and cytoplasmic CE hydrolytic activities. Detailed analysis of enzyme activation showed that the CE cycle is altered, resulting in cytoplasmic CE accumulation[20–22] (Figure 2).

To define some of the regulatory mechanisms associated with the control of the CE cycle, including cytoplasmic CE hydrolase, in herpesvirus-infected cells, the level of CE hydrolase activation in MDV-infected cells was examined in the presence of: 1) dibutyryl cyclic adenosine monophosphate (AMP), 2) dibutyryl cyclic AMP added together with protein kinase, or 3) agonists of adenylate cyclase.[21] Activation of cytoplasmic CE hydrolase activity by cyclic AMP or protein kinase A was blocked in MDV-infected cells but not in uninfected cells or in cells infected with a control virus, turkey herpesvirus (HVT).[21] Furthermore the rate of cholesterol efflux from arterial smooth muscle cells challenged with dibutyryl cyclic AMP was unchanged in MDV-infected cells as compared with uninfected or HVT-infected cells, in which efflux was actually increased.[20,21] Hence it has been proposed that the reduced cytoplasmic CE hydrolase (NCEH) activity in lipid-laden, herpesvirus-infected cells was due in part to the inability of the enzyme to be activated by the cyclic AMP–protein kinase A mechanism.[21] This may contribute to the pathologic changes seen in MDV-infected arterial cells, such as intracellular CE accumulation.

We extended these studies to human arterial smooth muscle cells infected with herpes simplex virus (HSV) type 1: HSV also induced accumulation of saturated triacylglycerols and CE in infected cells.[22] The infected cells had reductions in the CE hydrolytic activities and in the enzyme itself, based on immunoprecipitation data (Figure 3) and activity assays because of decreased translation of the RNA that encodes the intracellular hy-

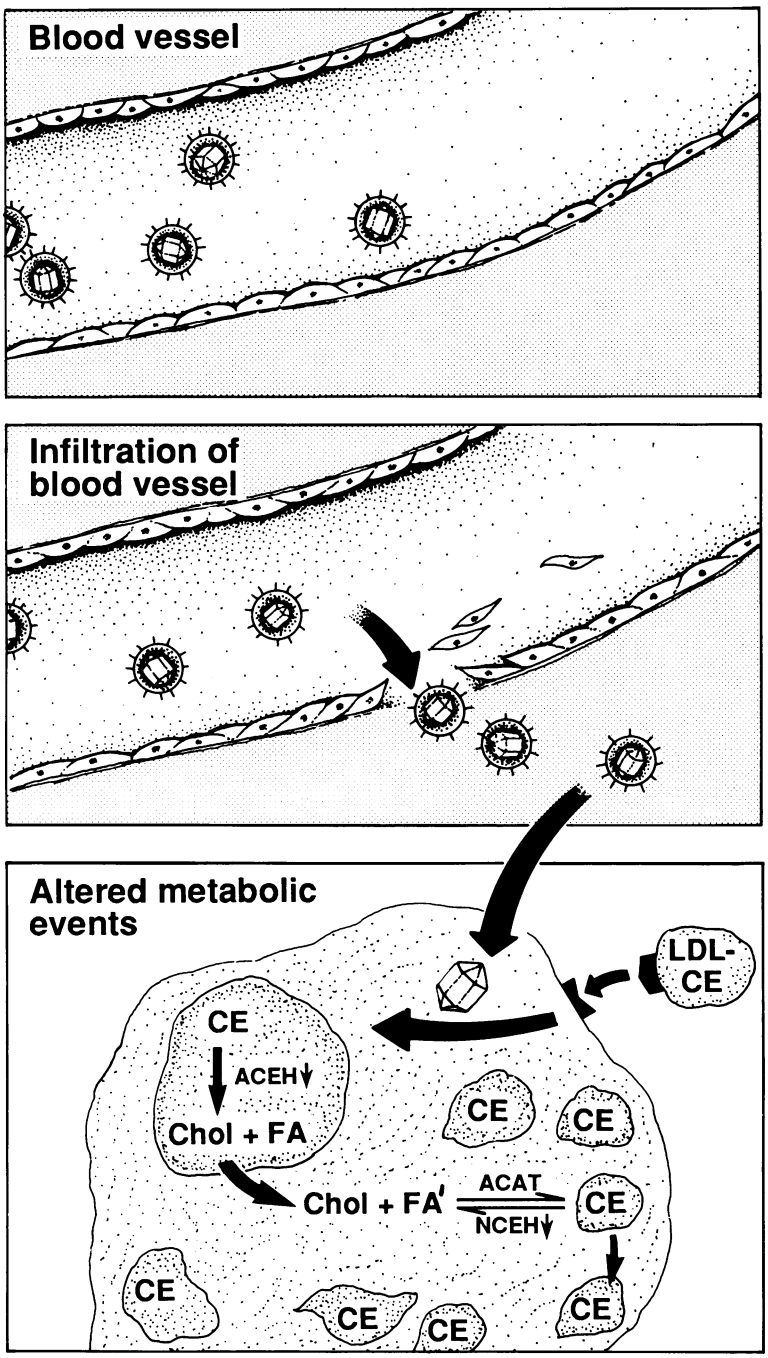

Figure 2. *Herpes virus penetration into cells leading to altered metabolic activity. This schema depicts a hypothetic sequence by which circulating herpes virus enters cells through the vessel wall and leads to altered metabolic activity. The intact circulating virus penetrates arteries by causing endothelial cell injury since these cells can be infected with the virus. Once the virions are shed to the arterial smooth muscle cell, increased binding of LDL-cholesteryl esters (CE) can occur, resulting in decreased CE hydrolase activities (ACEH and NCEH). This can lead to increased CE accertion in the cell in the form of CE-enriched lipid droplets, which are a characteristic feature of the atherosclerotic foam cell. Reprinted with permission.*[96]

drolases.[23] Because arachidonic acid metabolites such as prostacyclin (PGI_2) and 12-HETE alter CE hydrolysis in arterial smooth muscle cells[3], we measured these eicosanoids in HSV-infected cells. We observed a reduction in both spontaneous (baseline) and arachidonate-induced release of the cyclooxygenase product, PGI_2 and a lipoxygenase product, 12-HETE.[22] Hence we believe that the herpesvirus-induced atherosclerosis results, in part, from alterations in metabolic control of CE trafficking by eicosanoids in vascular cells. This concept is depicted in Figure 4.

Role of Cytokines in Cholesterol Trafficking in HSV-infected Cells

Recent studies have suggested that the immune system may participate in atherosclerosis, because monocytes and T lymphocytes accumulate within the atherosclerotic lesion.[2] Viral infection could induce mediator release from immune and hematopoietic cells, which may be responsible for phenotypic changes in vascular cells. Of interest in this regard are recent studies that show that herpesviruses are capable of inducing messenger RNA expres-

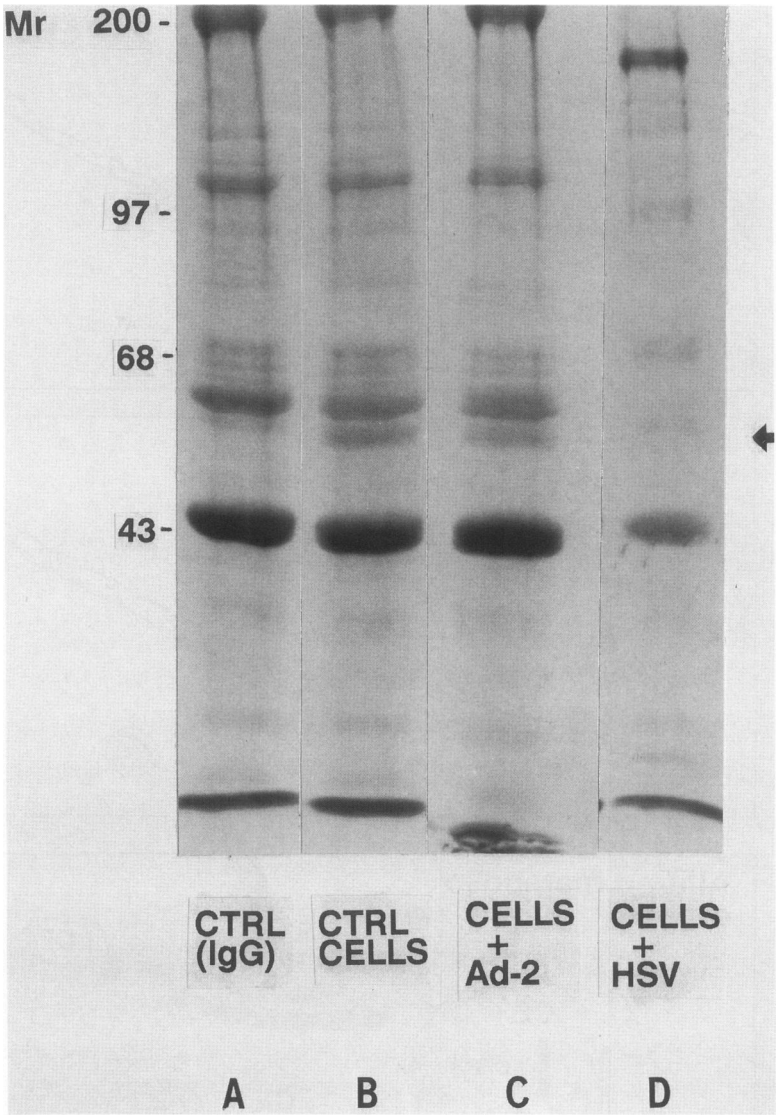

Figure 3. *Immunoprecipitation of acid cholesteryl ester hydolase ACEH from herpes virus infected cells. Immunoprecipitation of ACEH was done from a 24-hour mock-infected and HSV-infected (moi = 0.1) SMC culture. Biosynthetic labeling of cell protein was done with [35-s]methionine (40 uCi per 5 × 10^5 cells). Each lane contained about 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. 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 monoclonal antibody. (mAB) A band is shown by the arrow corresponding to M_r 55,000. Lane C, immunoprecipitated ACEH from adenovirus-infected cells (DNA virus control group). Lane D, 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 C) were higher than the amounts shown in lane D. We also observed a prominent protein band appearing at M_r 150,000 in lane D only, which may be of herpes viral origin. Finally, we assayed ACEH activity after immunoprecipitation in another experiment to confirm that we immunoprecipitated ACEH. We found that we could immunoprecipitate about 60% of the ACEH in the cell based on our activity found in the supernatant and pellet. Reprinted with permission.*[23]

sion of monocyte-derived genes. For example, CMV infection *in vitro* induces macrophages to increase the expression of specific RNAs for interleukin-1β (IL-1β), tumor necrosis factor (TNF), and colony stimulating factor.[43] The role of TNF in the complex cytokine network in mammalian cells is not fully understood, particularly regarding its antiviral activity. Although TNF is an inducer of interferon messenger RNA transcripts, a well-documented cytokine that has antiherpesviral activity, others have shown that TNF's antiviral activity is not abolished in the presence of antiserum to interferon.[44] Wong and Goeddel[45] have postulated that the antiviral activity of TNF is attributable to lysis of virally infected cells rather than to induction of interferon. Other possible mechanisms to explain the action of TNF involve eicosanoid metabolism, since TNF's lytic effects can be partially abolished with cyclo-oxygenase inhibitors. Such studies suggest that cytokines, such as TNF, can promote synthesis of eicosanoid products that, in turn, can mediate specific biologic effects such as modulation of cholesterol metabolism, a concept originally proposed by our laboratory.[46]

Because HSV infection of vascular cells can produce a biochemical and cytopathologic effect virtually indistinguishable from atherosclerosis, we hypothesized that these cytokines can prevent CE accretion in arterial smooth muscle cells that is associated with herpesvirus-induced atherosclerosis.[46] Tumor necrosis factor and IL-1, but not interferon, prevented CE accumulation in HSV-infected cells by induction of cyclic AMP–dependent CE hydrolysis. This effect was mediated through the arachidonate 12-lipoxygenase pathway by 12-HETE, because pretreatment of cells with a cocktail of lipoxygenase inhibitors abolished the antiviral effect and 12-HETE production in the cell.[46] 12-HETE is the major lipoxygenase

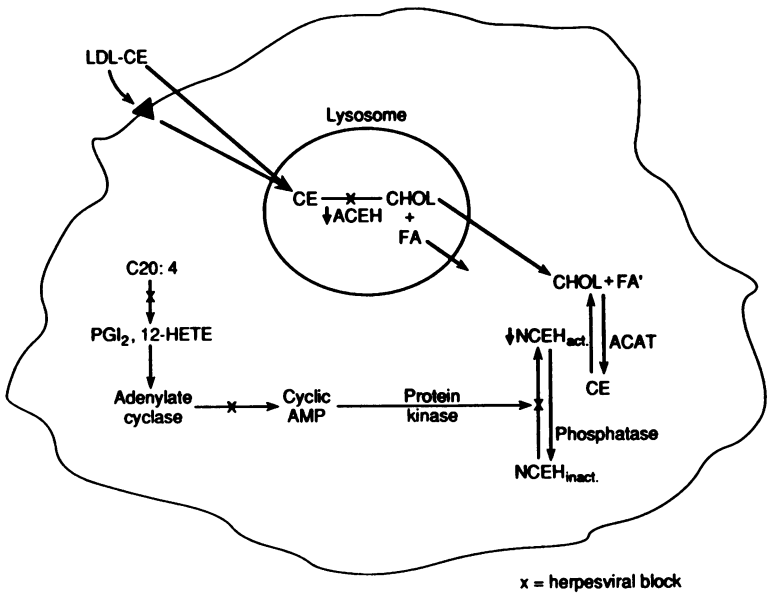

Figure 4. *Herpes virus infection blocks signal transduction pathways in arterial smooth muscle cells. This model shows that herpes virus infection leads to decreased lysosomal hydrolysis of CE by the ACEH enzyme. In addition, infection leads to decreased conversion of arachidonic acid (C20:4) to PGI_2 and 12-HETE, which in turn can lead to a reduced activation of adenylate cyclase. Decreased cyclic AMP production results in the cell. Continuing in this altered metabolic sequence of events, decreased cyclic AMP induces less activation of protein kinase A, thus reducing the activation (through phosphorylation) of the cytoplasmic CE hydrolase (NCEH) to the active form from the inactive form. These metabolic events in the cytoplasm, coupled to those in the lysosomes, results in intracellular CE accretion.*

metabolite found in arterial smooth muscle cells.[46] This overall conclusion is further supported by data that show that TNF and IL-1 enhance 12-HETE production, which in turn increases both intracellular cyclic AMP levels and CE hydrolysis.[46] Collectively these findings identified for the first time a biochemical mechanism involved in a cytokine-induced reduction of lipid accumulation in herpesvirus-infected arterial smooth muscle cells. This is potentially an important finding regarding control of cholesterol metabolism during viral-induced atherosclerosis, because cytokines are important regulators of intracellular lipid metabolism, not only in arterial smooth muscle cells,[46] but also in monocyte-derived macrophages.[47]

Potential Links Between Atherosclerosis and Thrombosis

A link between the coagulation system, fibrinolysis, and atherosclerosis has been proposed on the basis of thrombotic processes at the level of the endothelium. The association between lesion progression, lipid abnormalities involving elevations of lipoproteins, including lipoprotein (a) [Lp(a)], and the fibrin deposition is now being broadly defined. Early studies of Greenland Eskimos indicated that their diet, enriched in polyunsaturated fatty acids of fish oil origin, had an impact on platelet function and eicosanoid production. It has been hypothesized that if the vessel wall is injured (or activated), for example, by herpesviruses, the endothelium can become prothrombotic, as evidenced by several cytopathologic events. These include enhanced thrombin generation and enhanced binding of platelets to endothelium,[48] decreased PGI_2 production,[48] enhanced tissue factor production, reduced thrombomodulin expression,[49] and an inhibition of endothelial cell synthesis of heparan sulfate proteoglycan,[19] which is closely related structurally to heparin, a complex polysaccharide that has anticoagulant activity.

An example of molecular mimicry that relates thrombotic processes to atherogenic events involves the recent observation of striking structural homology between the apoprotein (a) component of human Lp(a) and plasminogen,[50] a major protein involved in fibrinolysis.[51] Increased levels of Lp(a) have been associated with atherosclerosis in humans.[50–52] Lipoprotein (a) as well as its apo(a) also have been shown to compete with plasminogen for cellular binding sites,[51] and Lp(a) has been associated with enhanced functional, antigenic, and transcript levels of plasminogen activator inhibitor (PAI) type 1 by the endothelium.[53] Plasminogen activator inhibitor type 1 is the rapidly acting physiologic inhibitor of both tissue-type and urokinaselike plasminogen activators. The net effect of these events leads to down-regulation of plasmin generation at endothelial cell surface.[53] Lipoprotein (a) appears to function under some circumstances as an inhibitor of plasminogen activators, by competing with plasminogen for binding to streptokinase or by acting as a competitive inhibitor of tissue plasminogen activator (t-PA) in the presence of fibrinogen. (KA Hajjar, personal communication). Recently we have observed that HSV infection may interfere with normal endothelial cell fibrinolysis, as is the case with Lp(a), by inhibition of plasminogen binding and increasing PAI-1 activity, thereby preventing plasminogen activation (unpublished observations). The mechanisms of action of HSV infection and

viral protein production on processes related to fibrinolysis are currently under investigation.

Viral Activation of the Coagulation Cascade

An important event in the early stages of atherosclerosis is the adhesion of blood cells to altered endothelium. Certainly both platelet (as well as neutrophil) and monocyte adhesion to the endothelium are processes attributable to the pathogenesis of thrombosis and atherosclerosis. Monocytes can migrate into the vessel media and begin accumulating cholesterol, contributing to foam cell formation.[2]

Herpes simplex virus-infected endothelium, as well as endothelium exposed to TNF or to IL-1, binds platelets,[48] granulocytes,[31] and expresses tissue factor to a greater extent than it does to noninfected cells.[49] Herpes simplex virus-infected endothelium also expresses glycoproteins encoded by the HSV genome.[54] These participate in molecular mimicry because of their presumed functional role on the endothelium. For example, glycoprotein E (gE) can function as an Fc receptor,[55–57] and glycoprotein C (gC) can also serve as a complement (C3b) receptor.[54] A role for these proteins in the pathogenesis of endothelial injury is suggested by the observation that polymorphonuclear (PMN) cell adhesion to HSV-infected endothelium can be blocked by antiviral serum.[58]

Central to the pathogenesis of vascular injury is the localized activation of the coagulation cascade and the adhesion of circulating inflammatory cells to the exposed subendothelial vascular surface. Once adherent, these cells can secrete growth factors, proteolytic enzymes, and cytokines that further activate the injured vessel surface.[2,3] For example, macrophage-derived cytokines such as TNF and IL-1 induce endothelial expression of leukocyte adhesion molecules[59] as well as tissue factor.[60] These events promote further leukocyte accumulation and localized thrombin generation.

Recently we demonstrated that infection of endothelial cells promotes enhanced monocyte adhesion.[61] Enhanced adhesion was blocked by monoclonal antibodies to the virally encoded cell surface glycoprotein g(C) but not by antibodies to g(D) or g(E). Adhesion also was blocked by treating endothelial cells with specific thrombin inhibitors or by growing cells in prothrombin-depleted serum. This suggested that thrombin plays a role in the enhanced monocyte adhesion. Glycoprotein C bound and promoted activation of factor X on infected endothelial cells, thereby contributing to thrombin generation. To further support the hypothesis that g(C) was indeed involved in monocyte adhesion and factor X binding, we used cells (L cells) that do not normally express this glycoprotein but could be transfected with the gene for herpes-virus glycoprotein C.[61] We found that factor X bound to transfected L cells that were induced to express g(C) by dexamethasone, a steroid hormone that activated the MMTV-LTR promoter region of an artificial g(C) gene construct. Cross-linking and immunoprecipitation studies demonstrated factor X–g(C) complex formation on the cell surface, suggesting that g(C)-dependent thrombin generation by herpes-infected endothelium may be an important mediator of vascular pathology during viral infection. Thrombin itself can elicit a variety of cellular events. These include monocyte and neutrophil adhesion, and cytokine release and platelet activation on a damaged surface.[48,60,61] Each one of these cytopathologic effects has been linked to the role of inflammation as it may present itself during the pathogenesis of atherosclerosis.

Identification of a Monocyte Receptor on Herpesviral-infected Endothelium

Endothelial cells express several leukocyte receptors, including GMP-140 (also known as PADGEM or CD 62), ELAM-1, ICAM-1 and 2, and VCAM-1 on their surfaces in response to cytokine or exposure to other agonists.[62] Normally GMP 140 is a cytoplasmic protein found in resting endothelial cells found on the membrane of Weibel-Palade bodies.[63] After stimulation by thrombin, histamine or complement proteins, the Weibel-Palade body is rapidly translocated, and its membrane becomes incorporated into the plasma membrane, resulting in surface expression of GMP 140.[63] This mechanism of new protein expression, ie, translocation from a preformed intracellular membrane compartment to the cell surface, does not require *de novo* protein synthesis. As described in the previous section, our recent data support the following model: HSV infection induces endothelial cell surface expression of HSV g(C), which acts as a binding site for factor X. Concomitant generation of tissue factor converts bound factor X to an active prothrombinase, leading to generation of thrombin in the microenvironment of the infection.[61] Recently we have extended this hypothesis, because we now have evidence that thrombin can act in an autocrine manner to induce expression of the leukocyte receptor GMP140.[64] Our data indicate that monocyte adhesion induced by HSV infection is blocked by anti-GMP140, but not by anti-ELAM or antibodies to other adhesion molecules. This suggests that GMP-140 is a major receptor for monocytes on the HSV-infected endothelium.[64] These findings are summarized in Figure 5, which shows that HSV-infected endothelial cells generate thrombin, which predisposes to increased monocyte adherence by expressing the monocyte receptor GMP-140. Thrombin generation by these cells is dependent on the

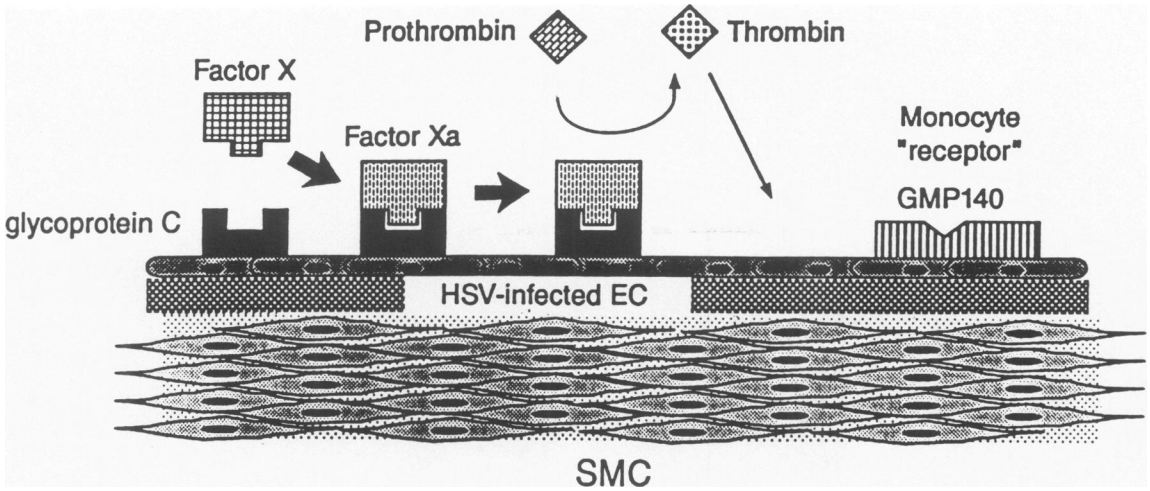

Figure 5. *Viral activation of the coagulation cascade. Hypothetical model depicting how herpes simplex virus (HSV) infection can lead to a prothrombotic, pro-atherosclerotic state on the endothelial surface by inducing the synthesis and expression of glycoprotein (C) which can serve as a binding site for Factor X, a key proenzyme of the coagulation cascade. This increased binding of Factor X and its subsequent conversion to the active form, Factor Xa, can lead to the conversion of prothrombin to thrombin (Factor IIa). When Factor IIa is generated, this can induce surface expression of a monocyte receptor (GMP-140), which in turn can promote monocyte adhesion to the endothium and induction of an inflammatory response by activating the cytokine network. Each of these cytopathologic feaures can predispose to atherosclerosis and thrombosis.*

expression of HSV g(C), which can act as a site for factor X binding and assembly of the prothrombinase complex. As stated earlier, other receptors do not play a major role in this cell system. For example, the leukocyte integrins (LFA-1, Mac-1, and p150,95) mediate adhesion to ICAM-1 and ICAM-2 on cytokine-stimulated endothelial cells,[65] but do not appear to play a role in adhesion to the virally infected cells, based on lack of inhibition seen with specific monoclonal antibodies or RGDS peptides.[64] Similarly VCAM-1, which mediates lymphocyte adhesion to stimulated endothelial cells,[66] is not functional in this system, based on lack of inhibition with anti-VCAM antibodies.[64]

The significance of a procoagulant phenotype of HSV-infected cells is considerable: local generation of thrombin at the site of infection may activate platelets as well as endothelial cells. Thrombin-stimulated platelets can adhere to monocytes, and therefore can recruit more platelets into the site of injury.[2,64,67] The presence of activated platelets and monocytes at the locus of infection may contribute further to the development of vascular injury, chronic inflammation, and atherosclerosis by way of release of cytokines and lipoxygenase products.[46,58,64] Expression of adhesion molecules on HSV-infected cells thus may be an initial step in viral-mediated endothelial injury and atherogenesis.

Most recently, to identify the putative cell-interacting sites in factor X, we have analyzed the structural domains of factor X that are involved in the coordination of its binding to membrane receptors.[68] A group of partially overlapping synthetic peptides representative of different regions of the factor X molecule were used. Two unrelated surface membrane receptor regions that coordinate this recognition have been identified: CD11b/CD18 on monocytes and glycoprotein C (gC) on HSV-infected endothelium, ie, they recognize a common structural motif in the catalytic domain of factor X.[68] Each of the peptides we used blocked factor Xa–mediated monocyte procoagulant activity and suppressed monocyte adhesion. The structure of the catalytic domain of factor X containing the three sites that are involved in binding to herpesviral g(C) and CD11b/CD18 was modeled computationally. Factor X associates itself with herpesvirus-infected endothelial cell and monocyte receptors using a similar molecular recognition motif. We were able to show that the vascular cell binding region of factor X is organized into three distinct interacting sites. The peptidyl analogs prevent the consequences of vascular generation of thrombin such as PMN adhesion to endothelial cells, chemotaxis, and monocyte-mediated deposition of insoluble fibrin. Our understanding of the cell biology of this system coupled with the molecular modeling offers an unprecedented paradigm of the molecular complexity of this thrombotic process that can be linked to viral-induced atherosclerosis; it also directly links the activation of coagulation proteins on vascular cells to typical inflammatory–thrombotic reactions. This was observed by constituting three spatially distant surface loops that define a unique three-dimensional cell-interacting network in the ligand, as shown in Figure 6.

In summary, we observed that specific synthetic peptides inhibit factor X binding to monocyte CD11b/CD18 and to g(C) expressed on herpesvirus-infected endothelial cells. These peptides blocked monocyte generation

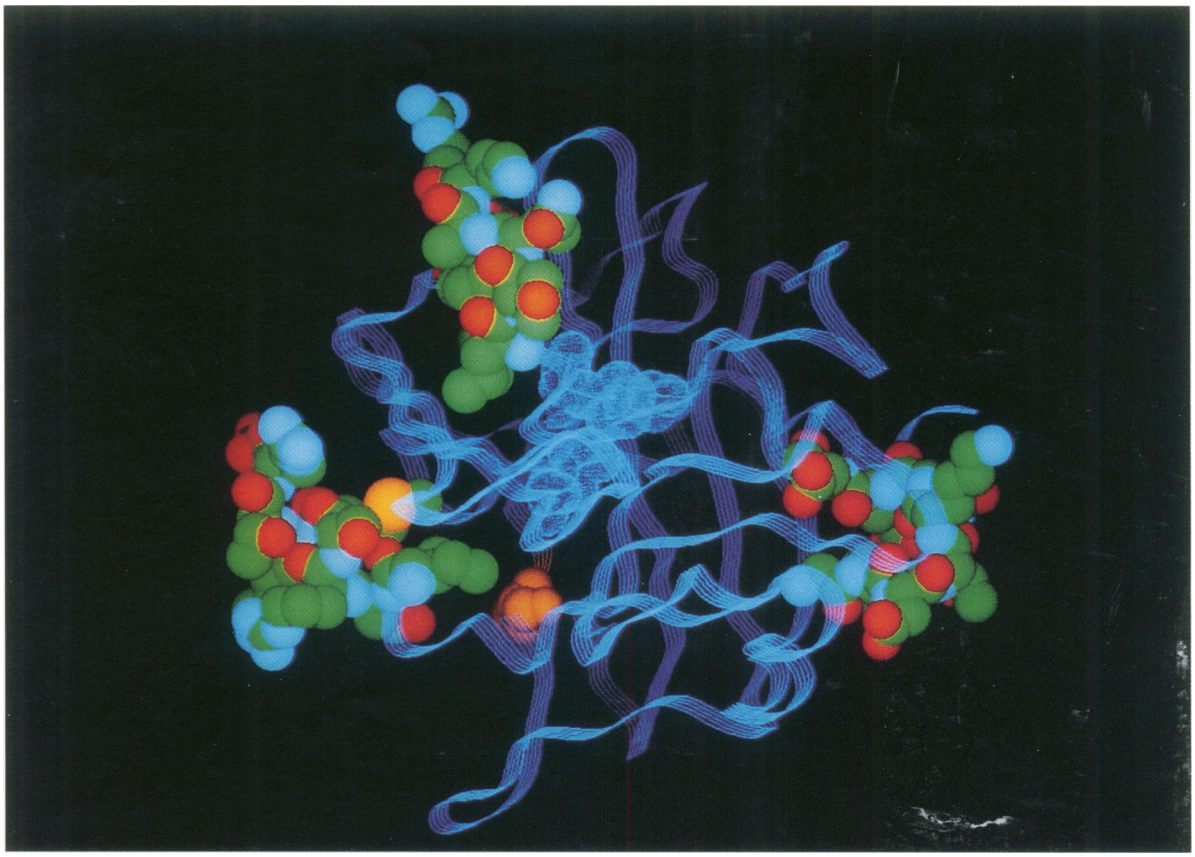

Figure 6. *Structural model of the Factor X catalytic domain. Homology model building techniques were used to construct a model of the catalytic domain of factor X from a crystal structure of trypsinogen. Structurally conserved regions were identified by visual inspection of the structures of trypsin, chymotrypsin, elastase and kallikrein, and the Factor X sequence was aligned manually with the sequence of these proteases. Atomic coordinates for backbone and conserved side chains of Factor X were assigned directly from trypsinogen for the structurally conserved regions. Coordinates of nonconserved side chains within structurally conserved regions were computed for minimal overlap with other atoms. Atomic coordinates for structurally variable (loop) regions were taken from a prior, unpublished model of Factor Xa (T. Edgington et al.). The initial model structure was energy minimized in stages, first allowing all side chains to relax, and then relaxing all side chains together with the backbone atoms of the loop regions. Backbone of Factor X catalytic domain, (ribbon diagram). Catalytic triad, blue van der Waal surface (center). Loop peptide 1 (GYDTKQED) (67) CPK surface (right). Loop peptide 2 (IDRSMKTRG) (67), CPK surface left. Loop peptide 3 (LYQAKRFKV) (67), CPK surface (top); Cys residue link to light chain, yellow CPK (lower center). The N-terminus of the catalytic domain is located just above peptide 1, the substrate binding groove is approximately vertical and to the right of the triad, and the C-terminus of the model (lacking 18 residues of Factor X sequences for which no structural information is available) is the C-terminus of peptide 2. Reprinted with permission.*[68]

of thrombin and prevented monocyte adhesion to HSV-infected endothelium. It is possible that selective interruption of coagulation by such synthetic analogs may impact positively to reduce vascular injury associated with monocyte adherence and HSV infection of the endothelium.

Molecular Genetics of Atherosclerosis

The monoclonal hypothesis of Benditt and Benditt[4] was based on the observation that 75% of atherosclerotic plaques from human tissues removed at surgery or at autopsy demonstrated a monoclonal phenotype of the glucose-6 phosphate dehydrogenase (G-6-PD) enzyme, based on electrophoretic mobility. This technique took advantage of the fact that the polymorphic forms of this enzyme are expressed in an X-linked fashion. Medial cells from regions adjacent to atherosclerotic plaques from nonplaque areas display equal amounts of each polymorphic form of the enzyme, as would be expected in an X-linked gene, because one of the two X chromosomes is inactivated early in embryonic development. Similar results have been obtained by other laboratories,[69,70] however the interpretation of these experiments in supporting the monoclonal hypothesis has been challenged.[71] The principal argument is that the G-6-PD marker may be linked to some other gene that gives these cells a selective growth advantage, ie, a 'phenotype-selective advantage' without the proliferative lesion being truly monoclonal in origin.

The monoclonal hypothesis purports that the smooth muscle cell proliferation seen in the atherosclerotic lesion is similar to a benign tumor, for example, a uterine leio-

myoma.[72] A logical extension of this hypothesis is the inference that alterations in the smooth muscle cell genome by chemical mutagens or viruses may be involved in the smooth muscle cell proliferation characteristic of human atherosclerosis. This thesis, in part, provided the rationale for testing the viral hypothesis in an animal model that would produce an arteriopathy approximating the human disease. Supporting such an idea is the experimental observation of focal smooth cell proliferation induced in the aortic intima of chickens treated with a chemical mutagen initiation–promotion sequence.[73]

To extend these observations to the level of the genome, Penn et al[74] reported the presence of transforming DNA in samples derived from plaques of human coronary arteries. Plaque-derived DNA but not DNA from normal human arteries had the capacity to transform NIH 3T3 cells *in vitro*. The transformed cell line was capable of forming tumors in nude mice. The original observations regarding the presence of a transforming gene element have been extended into an animal model of early arterial plaques induced by administration of the carcinogen 7,12, dimethylbenz(a)anthracene to cockerels.[75] Further studies on vascular smooth muscle derived from human plaques show enhanced expression of the proto-oncogene c-*myc*.[76] The concept that increased expression of this transforming gene is a generalized phenomenon in atherosclerotic lesions has been challenged recently by Yew et al,[77] who were unable to detect transforming activity in DNA isolated from atherosclerotic plaques of human carotid arteries. Nevertheless other laboratories have detected a similar transformed phenotype in cells derived from atherosclerotic lesions.[78] This recent study reported the correlation of transformation with the absence of a 140-kd protein normally secreted by these cells. Other studies have identified a 140-kd antitumorigenic glycoprotein that acts as an anti-angiogenesis factor.[79,80] The loss of this protein correlates with a transformed phenotype. Taken together, these findings raise the possibility that the transforming gene activity may represent the loss of a normal anti-oncogene or a gene controlling cellular proliferation. Precise identity of the transforming gene or genes remains unidentified.

Although no transforming gene of viral origin has been identified to induce human atherosclerosis, the results of recent studies suggest that vascular cells have the potential to be transformed by viral genes. Natchtigal and colleagues[81,82] have shown that vascular smooth muscle cells can be transformed after transfection with a plasmid containing the BglII N fragment (MTRII) of HSV-2. Immortalization is probably the most important attribute of transformation, because these cells normally have a finite life span *in vitro*.[81] The transformed cells did not retain the viral DNA sequences, consistent with other models of HSV-induced transformation. Interestingly transformation of rat embryo cells by SV40 can result in marked accumulation of CE, apparently because of an alteration in the regulation of low-density lipoprotein receptors.[83] Thus these studies provide circumstantial evidence that virally induced transformation can predispose to some of the characteristic features of atherogenesis. More studies are necessary to prove that the vessel wall actually contains transformed cells that are part of the foam cell population in atherosclerotic plaque regions. In Figure 7, I have summarized three hypothetical cytologic alterations induced by herpesvirus infection. The atherogenic potential of viruses in acute infection, chronic infection, with ongoing immunologic activation, and transformation of vascular smooth muscle cells is depicted.

The mechanism of transformation by herpesvirus DNA fragments has not been elucidated. Proposals have been made to implicate many factors, including DNA stem loop structures, ribonucleotide reductase activity, mutagenesis, gene amplification, increased or altered expression of cellular genes, activation of endogenous viruses, protein kinase activity, and the possible interaction between latency of the virus and transformation.[84–86] No conclusive proof exists for any of these mechanisms. Recently shuttle vectors have been employed to study sequence analysis of the mutations induced in cellular DNA by HSV-1.[85] Interestingly the maximal increase in mutation frequency was noted at 4 hours after HSV-1 infection, implicating an immediate–early gene or early viral protein. Sequence analysis of the chromosomal fragments may lead to information about putative cellular targets for the virally induced mutagenesis. Mutagenesis may be the explanation for a 'hit and run' mechanism of virally induced transformation.[86] Because vascular smooth muscle cells can be transformed by HSV-2,[82] herpesvirus-induced transformation may be a contributory mechanism to periods of uncontrolled growth in the atherosclerotic vessel wall. This is an attractive proposal and needs to be explored experimentally.

Herpesvirus Entry into Vascular Cells

After several decades of investigation, the pathway of herpesviral entry into cells still remains undefined, partly because of the complexity of the virion. Herpes simplex virus is a large DNA virus whose nucleocapsid is surrounded by a lipid envelope. Seven herpesvirus-encoded glycoproteins (termed gB, gC, gD, gE, gG, gH, and gI) have been identified on the envelope.[87] Four of these, g(B), g(C), g(D), and g(H), appear to play a role in infectivity. Three glycoproteins, g(B), g(D), and g(H), are essential for infectivity and appear to be involved in the process of viral penetration.[87] The process pathway by

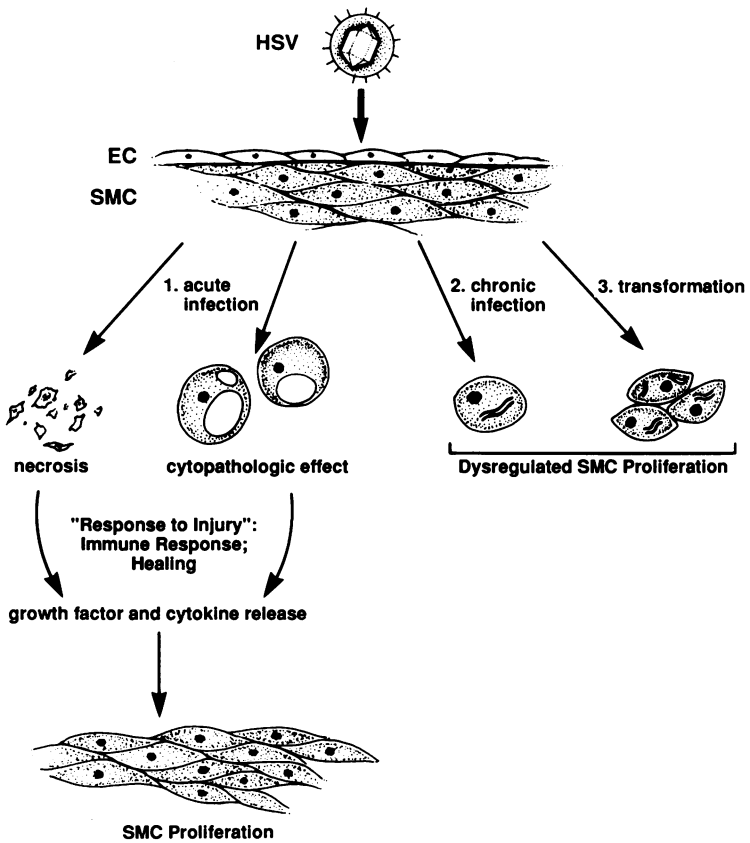

Figure 7. *Three cytologic alterations induced by herpes virus infection. A hypothetic model depicting viral infection of the vessel wall which can induce three cytopathologic states:* acute infection, *which can lead to altered cellular morphology, lipid accumulation, altered extracellular matrix, altered functional properties of the vascular cells and acute inflammation, with growth factor and cytokine release, possibly resulting in smooth muscle cell proliferation;* chronic infection, *which could provide the immunologic stimuli for an ongoing cell-mediated inflammatory response with subsequent cellular infiltration of the vessel wall leading to dysregulated smooth muscle cell proliferation; or* transformation, *which leads to increased proliferation since the cells lose their capacity to downregulate growth and cell division appropriately. Reprinted with permission.*[96]

which herpesviruses enter vascular cells to initiate an infection, however, has not been completely characterized. We have become interested in the pathway of herpesvirus entry into vascular cells because the virus can induce arterial injury or activation associated with lipid accretion and atherosclerosis, as discussed in previous sections.

WuDunn and Spear[88] have shown that the initial attachment of virions to the cell surface occurs when the virus binds to heparan sulfate. The virion envelope glycoproteins involved in this attachment may include g(B) and g(D) or g(C). Recent studies in our laboratory,[89] and in collaboration with others,[90] suggest that after this initial interaction, HSV-1 may use a basic fibroblast growth factor (FGF) receptor to enter some bovine and human endothelial and smooth muscle cells. Herpesviral infectivity was partially blocked (<70%) when the high-affinity FGF receptor was blocked using FGF peptides that bind to the high-affinity FGF receptor. Not all vascular cells, such as the rat arterial smooth muscle cells, appear to use an FGF receptor complex as a major portal of entry for HSV (preliminary observations). The reasons for this are unclear. We speculate that receptor density or receptor conformation on the cell surface in specific animal species may alter the mode of viral penetration. In bovine cells, for example, we did observe that the virus appeared to recognize the receptor, because our viral preparations contained basic FGF. We believe that the virus uses FGF from the cell or matrix to penetrate other host cells (Figure 8B). Of interest is the observation by Baird et al90 that HSV preparations are capable of inducing phosphorylation of a 90-kd substrate that represents a specific intracellular response to basic FGF through activation of its receptor.[90] An alternative hypothesis is that viral glycoproteins interact directly with the receptor, perhaps because it may share some structural homologies with basic FGF (Figure 8A). One of the envelope glycoproteins, g(D), is required for viral entry into cells, as evidenced by the following: 1) Monoclonal antibodies that react specifically with g(D) inhibit virion uptake.[91] 2) Mutant viruses lacking g(D) can adsorb to the cell surface, but cannot penetrate.[92] 3) UV-inactivated virions containing g(D) are capable of blocking entry of HSV-1, whereas a similar quantity of inactivated virions lacking g(D) cannot inhibit virus entry.[92]

The epitopes involved in g(D) function have been mapped with the use of deletion mutants and complementation assays.[93,94] Specific mutations in HSV-1, g(D) have been shown to correlate with neuroinvasiveness.[95] Because g(D) is essential for virus penetration,[92] this property may be due to the ability of the virus to penetrate cells of the peripheral nervous system and other organ systems. Whether mutations in either the virion, cellular

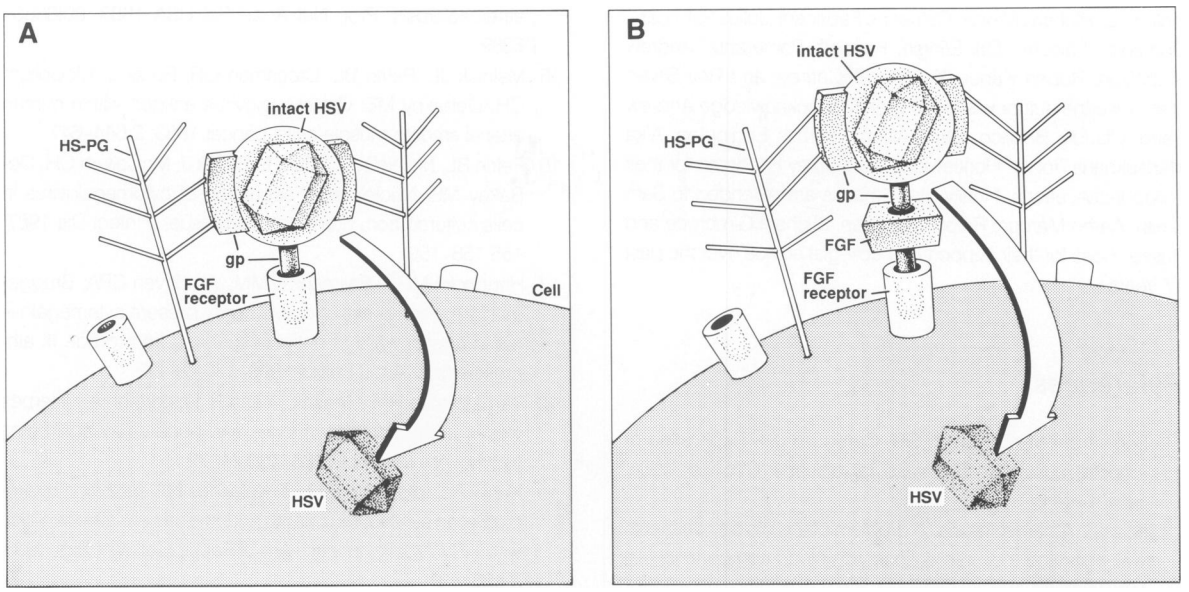

Figure 8. *Possible mechanisms of HSV penetration into vascular cells by the basic fibroblast growth factor (FGF) receptor. Two models are presented by which herpes simplex virus (HSV) type 1, may penetrate vascular cells.* **A**: *Intact HSV binds directly to the basic FGF receptor owing to the presence of several major glycoproteins (gp) such as gp(B), gp(C), gp(D), and/or gp(H) after adsorption to the surface of cells by heparan sulfate proteoglycans (HS-PG). Molecular mimicry between these viral glycoproteins and portions of the FGF receptors may exist. These HS-PG may also serve to stabilize the HSV complex on the surface of the cells by interacting ionically with the glycoproteins; the virus then penetrates the cell through the FGF receptor;* **B**: *Intact HSV "piggy-backs" on FGF, the natural ligand for the FGF receptor, after adsorption to the cell via HS-PG. FGF binds to its receptor directly and is internalized into the cell, dragging HSV along with it into the cell. In both scenarios, once the virus penetrates the cell, it then begins its replication process. Reprinted with permission.*[96]

receptors, or their expression will alter the susceptibility to infection of individuals and their arteries are interesting concepts, but they remain to be explored. From a developmental aspect, the concept that herpesviruses may use, in part, a growth factor or its receptor to penetrate cells *in vivo* is very intriguing because FGF levels in the cell have been linked to processes related to cell differentiation, cell division, and aging that are often associated with human atherosclerosis.

Summary

Circumstantial evidence continues to accumulate to support the hypothesis that certain herpesviruses can contribute to specific pathogenic events associated with atherosclerosis. This includes 1) evidence of widespread infection of herpes viruses in the general population, wherein these viruses are found in the arterial wall, including lesion-bearing areas; 2) the recognition of the association between accelerated atherosclerosis and CMV infection in cardiac allograft recipients; 3) the demonstration of herpesvirus-induced atherosclerosis occurring in animal models of the disease during normocholesterolemia; 4) profound effects on cholesterol metabolism in human, bovine, and avian arterial smooth muscle cells infected with herpesviruses, predisposing to free and esterified cholesterol accumulation; 5) alterations in extracellular matrix and enhancement of a procoagulant milieu on vascular cells infected with herpesvirus *in vitro;* 6) induction of monocyte and PMN adhesion receptors on HSV-infected endothelial cells; 7) transforming potential of herpesviruses and the ability of vascular smooth muscle cells to be transformed by such viruses; and finally, 8) the ability of herpesvirus to induce activation of specific cytokine genes that have been implicated in the growth regulation of the vessel wall during human atherogenesis.

Although the relationship of herpesviruses and atherosclerosis in humans is not unequivocally direct, data are accumulating that indicate that specific viral vectors may indeed have a participatory role in the induction of arterial injury. This also includes activation of the coagulation cascade with links to thrombotic processes and development of a complex arteriopathy. With the advent of the application of new molecular techniques to study transforming events in the arterial wall, undoubtedly new information will emerge regarding the viral origin of atherosclerosis.

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