Developmentally Regulated Herpesvirus Plaque Formation in Arterial Smooth Muscle Cells

Robert J. Kaner, John Medina, Andrew C. Nicholson, Roxana Ursea, Stephen M. Schwartz, David P. Hajjar

Previous studies of age-related susceptibility to viral infection have focused largely on the effects of aging on the immune response. Little attention has been given to age-related changes in the infectivity of target cells. We show here a fourfold greater plating efficiency of herpes simplex virus type 1 (HSV-1) for cultured vascular smooth muscle cells derived from adult rats compared with cells from genetically identical pup rats. The difference in plating efficiency appeared to be due to differences in initial attachment of the virion to the cell surface. There were no differences in the rate of viral entry or the efficiency of viral replication at high multiplicities of infection and no resistant “subpopulation” of pup cells. The pup cells did not release a soluble inhibitor of infection. Infection in both cell types was inhibited similarly by basic fibroblast growth factor (bFGF). Although adult cells exhibited a more vigorous mitogenic response to bFGF than did pup cells, binding studies did not demonstrate significant differences in the binding of bFGF to the cell surface, suggesting that differential expression of high-affinity FGF receptors could not be correlated with the difference in infectivity. We speculate that differences in the distribution of heparan sulfate in the cell surface, which serves as the initial attachment site for HSV-1, may explain the observed differences in plating efficiency. Since age is a risk factor for the development of atherosclerosis, these results have potential implications for susceptibility of the vasculature to herpesviral infections as a function of the development of the vessel wall. (Circulation Research 1993;73:10-14)

Key Words • smooth muscle cell • herpes simplex virus type 1 • developmental regulation • viral plating efficiency

Viral infection of atherosclerotic lesions has been demonstrated in human tissue,1,2 and infection with Marek’s disease virus has been shown to cause an atherosclerosis-like disease in chickens.3,4 Although neither the avian studies nor the presence of virus in human lesions proves that viruses cause human atherosclerosis, such an etiology is of interest for two reasons. First, viral infection of smooth muscle cells can mimic some of the abnormal properties associated with atherosclerotic smooth muscle cells,5,6 such as cholesterol ester accumulation; second, viral initiation of lesions offers a plausible explanation for lesion distribution and apparent monoclonality.7

If viral infection is an initiating event in the formation of human atherosclerotic lesions, then the viruses must be tropic for intimal smooth muscle cells. Although medial smooth muscle cells can show many of the degenerative changes of atherosclerosis, the formation of focal smooth muscle masses is specific for the intimal smooth muscle in humans just as it is in the chicken model described above. Such a tropism would suggest that intimal smooth muscle cells are unique in some way that is permissive for viral infection. Indeed, we know that intimal smooth muscle cells have several properties that distinguish them from medial smooth muscle in vivo.8,9 Moreover, distinct properties of intimal and medial cells have also been described in vitro, suggesting that intimal smooth muscle cells represent either a different developmental stage or a distinct smooth muscle lineage.10,11 This possibility is further strengthened by observations that cultured cells from the neointima of balloon-injured vessels and cultured cells from the walls of 2-week-old arteries overexpress several genes that are either absent or expressed at much lower levels in cells derived from the adult artery.12 The present study was undertaken to determine if developmental differences in smooth muscle could produce tropisms that would be specific for different vessel wall layers.

Previous studies of age-related susceptibility to viral infection have emphasized age-related changes in the immune response,13 including the state of macrophage activation,14,15 and in the release of interferon.16 Studies of age-related changes in viral receptors as a mechanism of age-related susceptibility to viral infection were inconclusive17 but have not been extensively investigated. The present study is the first to address age-related changes in susceptibility of vascular cells to viral infection.

Materials and Methods

Cells and Virus

Wistar-Kyoto (WKY) rat vascular smooth muscle cells were obtained from explants of thoracic aorta, as previ-
ously described. Cells were propagated in Waymouth culture medium with penicillin (100 U/mL), streptomycin (0.1 mg/mL), amphotericin B (2.5 μg/mL), and 5% fetal calf serum (FCS). African green monkey kidney (VERO) cells were obtained from American Type Culture Collection (ATCC) and cultured in medium 199 (M199) containing penicillin (100 U/mL), streptomycin (0.1 mg/mL), amphotericin B (2.5 μg/mL), and 10% FCS. Herpes simplex virus type 1 (HSV-1) strain F was obtained from ATCC and propagated in VERO cells.

**Binding Assays**

Cells were plated at subconfluent density in 24-well plates 24 hours before the experiment. 125I-labeled fibroblast growth factor (bFGF) was incubated with cells in the presence or absence of excess cold bFGF at 4°C for 2 hours. Wells were washed three times with cold phosphate-buffered saline (PBS), followed by a 2 M NaCl wash. Radioactivity was assessed by gamma spectrometry in the 2 M NaCl wash and the cell layer after lysis in 0.2 M NaOH.

**Mitogenesis Assays**

Cells were seeded into 96-well plates at a density of 10,000 cells per well. After 24 hours, the growth media was changed to media without fetal FCS containing 1% insulin-transferrin-selenium (ITS) in order to growth-arrest the cells. bFGF (purified human recombinant, a generous gift of Dr Andrew Baird, Department of Molecular and Cellular Growth Biology, The Whittier Institute, La Jolla, Calif), platelet-derived growth factor (PDGF, R&D Systems, Inc, Minneapolis, Minn), endothelial cell growth factor (EGF, Molloy), or FCS (GIBCO, Grand Island, NY) were added 24 hours after the cells were growth-arrested with serum-free media containing 1% ITS. The following day, the cells were pulsed with [3H]thymidine, and the incorporated radioactivity was collected on a cell harvester (Mini-mash) and counted in a scintillation counter.

**Viral Plaque Assays, Including Conditioned Media Experiments**

Confluent monolayers of cells grown in 12-well plates were infected with 1000 plaque-forming units per well of HSV-1. For conditioned media experiments, the infecting suspension was mixed with an equal volume of conditioned media collected after 24 hours of incubation with near-confluent cells growing in T-75 flasks. After 2 hours of adsorption at 37°C, the cells were washed three times with PBS and overlaid with 2 × M199 containing antibiotics and FCS mixed 1:1 with agarose 1.2%. The cells were incubated for an additional 48 hours, and plaques were counted in the light microscope after staining with methylene blue. The results of methylene blue staining were confirmed in cells stained with a polyclonal anti-HSV antibody (Dako Corp, Santa Barbara, Calif) that were grown in slide chambers and infected in a similar fashion.

**Viral Replication**

Smooth muscle cells grown in 12-well plates were infected with HSV-1 at a multiplicity of infection (MOI) of 1 for 2 hours. The cells were washed three times with PBS, refed with fresh media, and maintained at 37°C. After 24 hours, the media were collected separately, and the cells were scraped, collected, and frozen one time. After thawing and sonicating, infected media or cell sonicates were serially diluted with growth media and allowed to adsorb to monolayers of VERO cells in 12-well plates for 2 hours at 37°C. The monolayers were then washed extensively with PBS, overlaid with 1.2% agarose/2 × M199, and incubated for 72 hours at 37°C, and plaques were counted in the light microscope.

**Viral Temperature Shift-Up Experiments**

Near-confluent smooth muscle cells were infected with crude preparations of HSV-1 and allowed to adsorb for 2 hours at 4°C. After adsorption, the cells were warmed to 37°C and maintained at that temperature. Subsequently, the cells were washed with acid PBS (pH 3) at varying intervals to inactivate any extracellular virus. The monolayer was overlaid with agarose/2 × M199 containing antibiotics and FCS. After an additional 48 hours at 37°C, monolayers were stained with methylene blue, and plaques were counted in the light microscope.

**Results**

Plaque formation was fourfold greater in WKY adult cells than in pup cells when infected with HSV-1 in vitro (MOI, <0.01) as shown in the Table. This increase in viral-plating efficiency was not due to a resistant subpopulation of pup cells, because >95% of the cells demonstrated positive staining with an anti-HSV-1 polyclonal antibody when infected with higher MOIs (data not shown). Viral replication was assessed by infecting equal numbers of cells at MOI of 1, followed by harvesting the cells and media separately at 24 hours after infection, sonicating the cells briefly, and then performing plaque assays using VERO cell monolayers as the targets to assess the number of infectious particles present (Table). At this higher MOI, there were no significant differences in the quantity of infectious virus recovered. Thus, the pup cells appear to have a competent capacity for viral replication. In addition, we observed no differences in the uptake of labeled sucrose or thymidine over 30 minutes by these two cell types (data not shown), suggesting that the difference in viral-plating efficiency was not due to a global difference in bulk transport processes across the cell membrane.

Previously, we showed that bFGF blocked HSV-1 plaque formation, suggesting a role for the bFGF recep-

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**Herpes Simplex Virus Type 1 Plating Efficiency and Replication**

<table>
<thead>
<tr>
<th>Ratio (relative to VERO cells = 1)</th>
<th>Inhibition by 100 nM bFGF (%)</th>
<th>Supernatant Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>0.27*</td>
<td>61</td>
</tr>
<tr>
<td>Pup</td>
<td>0.07*</td>
<td>76</td>
</tr>
</tbody>
</table>

HSV-1, herpes simplex virus type 1; bFGF, basic fibroblast growth factor. Values are mean comparisons from three separate experiments with each condition done in triplicate.

HSV-1 plating efficiency was determined in a plaque assay with equal numbers of plaque-forming units added to each well. HSV-1 replication was expressed as the number of plaques per milliliter as determined by plaque assay of serial dilution of cell sonicates or supernatants on VERO cells. *P < 0.05 adult vs. pup by Student's t test.
Fig. 1. Bar graph showing mitogenesis as assessed by incorporation of [3H]thymidine into DNA. Smooth muscle cells from Wistar-Kyoto (WKY) rats plated in 96-well plates were growth-arrested in serum-free media containing 1% insulin-transferrin-selenium followed by treatment for 24 hours with endothelial cell growth factor (ECGF, 2 μg/mL), basic fibroblast growth factor (bFGF, 10 ng/mL), platelet-derived growth factor (PDGF, 10 ng/mL), or 10% fetal calf serum (FCS) as a positive control. After a 4-hour pulse with [3H]thymidine, cells were collected on an automated cell harvester, and incorporated radioactivity was quantitated in a scintillation counter. Each bar represents (mean ± SD) the counts from a single well (*n* = 6 per condition). Overall analysis of variance F ratio was 97.97 (*P* < .0001). Significant differences between the mean of each group and its respective control are indicated by letters *a* and *b* for *P* = 0.05, by Fisher's least significant difference comparison. Within each treatment group, differences between pup and adult are significant, except for PDGF.

Fig. 2. Graphs showing effect of donor age on basic fibroblast growth factor (bFGF) binding to Wistar-Kyoto rat smooth muscle cells. 125I-bFGF was incubated with or without excess cold bFGF at 4°C for 2 hours on subconfluent cells at 50,000 cells per well. Wells were washed three times with cold phosphate-buffered saline, followed by subsequent washes with 2 M NaCl. Radioactivity was counted in the washes and the cell layer in a gamma counter and expressed as counts per minute per well. Low-affinity binding (panel B) represented the radioactivity in the 2 M NaCl washes, whereas high-affinity binding (panel A) represented the cell-bound material after the 2 M NaCl washes.

more, whether the adult cells were serum-starved or actively dividing did not materially affect their viral-plating efficiency (data not shown).

To determine if treatment with bFGF was affecting the rate of viral entry into the cells by a mechanism other than interference with adsorption, we performed temperature shift-up experiments. As a control, we used antithrombin III, a heparin-binding protein, which does not block the effects of bFGF on [3H]thymidine incorporation. We observed that both bFGF and antithrombin III inhibited plaque formation (Figs 3A and 3B), because the plateau of these curves was lower than with the control cells. The slope of the initial phase of the curve was not greatly affected, suggesting interference with viral adsorption as opposed to subsequent entry. Inhibitory effects on the time course of viral entry after temperature shift-up in the presence of these two proteins were virtually superimposable (Fig 3).

Finally, we ruled out the possibility of the pup cells producing a soluble inhibitor of infection, since conditioned media from these cells did not inhibit infection in WKY adult or VERO cells (Fig 4).
FIG 3. Time course of penetration of herpes simplex virus type 1 (HSV-1) into rat smooth muscle cells. bFGF, basic fibroblast growth factor; AT-III, antithrombin III. The effects of bFGF and AT-III on the rate of HSV-1 entry were determined. To obtain comparable numbers of plaques, four times more input virus was used in pup cells relative to adult cells. Within each cell type, equal numbers of HSV-1 plaque-forming units were adsorbed at 4°C. At 0 minutes, the temperature was increased to 37°C, and at the time indicated, the cells were washed with acid–phosphate-buffered saline (pH 3) to inactivate any extracellular virus, and the cells were covered with agarose overlay. Plaques were scored at 48 hours after infection. Panel A: Wistar-Kyoto adult cells. Panel B: Wistar-Kyoto pup cells (four times input virus).

Discussion

It has previously been hypothesized that adult vascular smooth muscle cells might be more susceptible to viral infection than pup cells. The data presented here support this hypothesis. Along with other differences between arterial smooth muscle cells derived from rat pups and adult rats, these cells also differ in their susceptibility to infection with HSV-1. The difference in infectivity appears to reflect a difference in their ability to bind HSV-1. Differences in viral adsorption cannot be accounted for by soluble heparan sulfate since conditioned media from pup cells did not inhibit infection in other target cells. Alternatively, we speculate that differences in cell surface heparan sulfate could account for the differences in susceptibility to HSV-1 infection. Qualitative differences in the subspecies of heparan sulfate should also be considered. Although our binding studies did not demonstrate significant differences in low-affinity binding of bFGF, the subspecies of heparan sulfate proteoglycan that will bind HSV is not clearly defined. The minimum requirement for viral binding appears to be a heparan sulfate oligosaccharide containing at least 10 monosaccharide units whose capacity to compete with viral binding increases with a higher degree of sulfation. This could include heparan sulfate sequences similar to those recently identified as bFGF binding, such as an oligosaccharide containing seven disaccharide units, 74% of which were iduronic acid 2-sulfate–N-sulfated glucosamine [IdoA(2-OSO3)α1,4GlcNSO3].

Although the reasons for the differences in infectivity of adult and pup cells remain unclear, the ability of HSV-1 to attach to and subsequently infect rat arterial smooth muscle cells appears to depend on cell surface heparan sulfate. Although bFGF can inhibit HSV-1 infection, this is probably not related to the high-affinity FGF receptor in rat arterial smooth muscle cells since antithrombin III, an unrelated molecule that also shows high levels of heparan sulfate binding, is as effective as bFGF in inhibiting HSV-1 infection. Moreover, the differences between pup and adult cells probably cannot be accounted for by differences in the high-affinity receptor since we found no differences between the two types of cells in RNA levels for the flg FGF receptor (data not shown) and since we found no differences in FGF binding (Fig 2).

These new observations parallel some recent observations of others who also reported that the flg receptor
does not appear to be involved in HSV uptake in VERO, bovine kidney (MDBK), human epidermoid carcinoma (HEp-2 and A431), human lung fibroblast (MRC-5), human neuroblastoma (SK-N-MC), Chinese hamster ovary, and rat skeletal muscle myoblast (L6) cells. Earlier observations made by Baird et al.30 that HSV-1 competes with bFGF for one of the FGF receptors have to be reassessed in light of subsequent developments in the biology of the bFGF receptor system. Low-affinity binding of bFGF by cell surface heparan sulfate is required for high-affinity bFGF binding and for receptor function.31-33 The two systems cannot be easily separated. Thus, the inhibitory effect of herpes simplex virus preparations on bFGF binding to the high-affinity receptor cannot be unambiguously interpreted in terms of discerning low-affinity binding (heparan sulfate) from high-affinity receptor binding, since the former is required in order to achieve the latter. In fact, it is most likely due to the presence of bFGF in HSV-1 preparations, as we have previously demonstrated.30

Our data suggest a favored viral tropism for cells of the adult phenotype. We would like to speculate that the ability of HSV-1 to infect cells derived from young and old animals in vitro may reflect in vivo differences that occur with age. We further hypothesize that viral infection of medial smooth muscle cells expressing the adult phenotype may initiate a process favoring the proliferation of smooth muscle cells expressing the pu/nointernal phenotype. If herpesvirus infection is an etiologic agent involved in the initiation of atherosclerosis,4-7,24 these differences could contribute to the age-related increased risk of disease.

Acknowledgments

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References

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