Viruses in the etiology of atherosclerosis

(human arteries/hybridization/DNA probe/herpesvirus)

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ABSTRACT To examine the possible role of viruses in the etiology of atherosclerosis, we searched for the presence of viral genomes in arterial tissues by in situ hybridization. Because chickens infected with Marek disease virus, a herpesvirus, develop atherosclerotic lesions after infection, we looked for the presence of herpesvirus or parts thereof in human arterial wall tissue, particularly in individuals with evidence of atherosclerosis. Herpesvirus probes were used on specimens of aortic wall removed from patients undergoing coronary bypass surgery. Evidence for the presence of herpes simplex viral mRNA was obtained in 13 specimens. Some of the specimens positive for herpes simplex virus appear to represent early stages in atherogenesis. Evidence for the presence of cytomegalovirus or Epstein–Barr viral genome was not observed in any of the specimens examined. We have also shown that herpes simplex virus can infect human fetal smooth muscle cells in culture. There are several ways in which viruses could operate in the pathogenesis of atherosclerosis: They could induce proliferation of artery wall intimal smooth muscle cells via injury or by genomic alterations leading to clonal expansion of intimal smooth muscle cell populations. We suggest that expression of at least a part of the herpesvirus genome in arterial smooth muscle cells may in some cases be instrumental in initiating or maintaining this enhanced cell proliferation. Furthermore, viral agents could explain other puzzling features in the occurrence of atherosclerosis and the attendant heart disease and strokes.

The causes of atherosclerosis are still a puzzle. Hypercholesterolemia, decreased high density lipoprotein cholesterol, hypertension and smoking, and risk factors for coronary heart disease and strokes appear to offer significant but only partial explanations of the severity of atherosclerosis (1). Studies of the pathogenesis of atherosclerosis have highlighted intimal smooth muscle cell proliferation as an early and critical event in the formation of raised lesions in the artery wall (2–5). The clonal character of the cell population of many atherosclerotic plaques (5–8) suggested new possibilities for etiologic factors in the development of plaques (6). Monoclonality of cell populations is found in several proliferative lesions known or presumed to be caused by viruses (9–11). We have chosen here to study the possibility that viruses may play a role in formation of atherosclerotic plaques.

Association of viruses with vascular lesions in chickens, suspected earlier (12, 13), has now been established by infection of specific pathogen-free birds with a variant of Marek disease virus (MDV); the arterial lesions produced resemble those of human atherosclerosis (14, 15). The fact that MDV is a herpesvirus directed our attention to this group of viruses as possible etiologic agents in human arterial disease. There are five known herpesviruses that commonly infect humans: herpes simplex virus (HSV) types 1 and 2, Epstein–Barr virus (EBV), cytomegalovirus (CMV), and herpesszoster virus. All five are widespread in human populations. They have the interesting property of persisting in a latent state after an initial infection and under some conditions of being reactivated to produce overt disease (16, 17). Furthermore, HSV, CMV, and EBV are able to induce cellular proliferation (18–20). Given these properties, together with the results from chickens infected with MDV, we decided to hybridize herpesvirus DNA and cloned DNA fragments to frozen sections of aortic tissue from patients undergoing coronary artery bypass surgery. Here we present preliminary evidence that herpes simplex viral genomes, or parts thereof, can be found in sections of human artery wall smooth muscle cells and in what appear to be early atherosclerotic lesions.

MATERIALS AND METHODS

Cells and Viruses. Cells were cultured in an atmosphere of 10% CO₂/90% air in Dulbecco’s modification of Eagle’s medium (Bio-Rad)/10% fetal calf serum on plastic tissue culture plates (Falcon). Human smooth muscle cells were grown from explants of abdominal and thoracic fetal aorta (21). HSV-2 strain 333 and the McIntyre strain of HSV-1 were propagated on monolayers of baby hamster kidney (BHK-21) cells infected at low multiplicity (0.01 plaque-forming unit per cell). CMV strain AD169 was propagated in Flow 5000 cells (Flow Laboratories), and EBV was obtained from cell line B95-8. Chicken embryo fibroblasts, grown from eggs from pathogen-free birds, and MDV-transformed lymphoblastoid cell lines MDCC-CU29 and MDCC-CU29 were kindly supplied by Julius Fabricant and grown in RPMI 1640 medium/10% chicken serum.

Plasmids. The construction and characterization of plasmids derived from pBR322 that contain fragments of the HSV-2 genome have been described (22). The 12 recombinant plasmids containing MDV DNA used in this study were a gift from Norman L. J. Ross (Houghton Poultry Research Station, England) who prepared them by cloning the HPRS 16/att strain of MDV into pBR322 (23). The EBV plasmid was the BamHI fragment V of the B95-8 strain of EBV (pDK14) and was the gift of Elliott Kieff. The plasmids were propagated in Escherichia coli strain HB101. Plasmid DNA was isolated by the cleared lysate technique (24) and purified by gradient centrifugation through CsCl.

In Situ Hybridization. Sections prepared in a cryostat and placed on glass slides were fixed and stored in ethanol at −20°C until required for hybridization. The preparations were dried in vacuum desiccators to remove all traces of ethanol. Subsequent treatments were as follows. In the first trials, 10 μl of probe was applied to each tissue section and the slide was covered with a washed silicone-treated coverslip. In a second set of trials a slightly modified protocol adopted from Godard and Jones (25) was used to better preserve cell constituents during in situ hybridization. Immediately prior to drying, slides were

Abbreviations: CMV, cytomegalovirus; EBV, Epstein–Barr virus; HSV, herpes simplex virus; MDV, Marek disease virus; pfu, plaque-forming units.
fixed in 0.1% glutaraldehyde for 20 min at 4°C and this was followed by hybridization.

Hybridization was carried out in 6X standard saline citrate (1X standard saline citrate = 0.15 M NaCl/0.015 M sodium citrate) at 68°C for 18 hr. After this period, coverslips were removed and the slides were washed extensively with large volumes of 2X standard saline citrate at 4°C, dried with ethanol, and coated with a 1:1 dilution of Kodak NTB2 emulsion in distilled water/1% glycerol. They were then dried and stored at 4°C for autoradiography. Slides were developed with Kodak D19, fixed in Kodafix, then stained in 10% Giemsa and examined for evidence of silver grains. Cells with 10 or more grains were considered positive when the background level was 1-3 grains per cell. For in vitro hybridization of cultured human arterial smooth muscle cells, the preparations were fixed in ethanol at -20°C, then hybridized as above.

Infection of Cells with Herpesviruses. Viral infection of the cultured smooth muscle cells was done as follows. HSV-1, HSV-2, CMV, and EBV obtained as indicated above were used. Human fetal abdominal aortic smooth muscle cells passaged six times after explant and grown to 60% confluency on glass slides were washed twice with phosphate-buffered saline; 40 μl of viral suspension was applied, adsorption was allowed for 45 min, then normal medium was added. Suspensions of HSV-1, HSV-2, CMV were at 1.25 × 10⁵ plaque-forming units (pfu)/ml; EBV in the supernatant from cell line B95-8 (not titrated) was used without dilution. Sets of cultures were fixed for hybridization at the following times after exposure to virus: HSV-1, 6 and 24 hr; HSV-2, 8 and 24 hr; CMV and EBV, 24 and 48 hr.

Plaque Assay of HSV-1 Human Smooth Muscle Cell Infection. Abdominal fetal aortic cells passaged six times after explant were grown to 60% confluency in 60-mm plastic dishes, washed three times with phosphate-buffered saline, infected with McIntyre HSV-1 at 1 pfu/cell without serum; adsorption was allowed for 1 hr, and cells were then cultured with normal medium until time of harvest. Cells were harvested by scraping, pelleted, washed once with phosphate-buffered saline, resuspended in 1 ml of Dulbecco's modified Eagle's medium, and freeze-thawed at -70°C three times. Plaque assay was done on BHK-21 cells grown to 60% confluency in 10-mm multiwell plates. One-tenth-milliliter portions of 1:10 serial dilutions of virus were adsorbed for 45 min, wells were then overlaid with 1 ml of 0.6% agarose/2X Dulbecco's modified Eagle's medium/2% fetal calf serum/antibiotics (1:1), and plaques were counted after 48 hr of incubation. As a control, Flow 5000 (Flow Laboritories) cells were similarly treated.

Arterial Specimens. Aortic buttons about 4 mm in diameter and of transmural thickness, removed from the anterior ascending thoracic aorta of persons undergoing coronary artery bypass surgery, were prepared by two procedures. Specimens in the first series, collected on gauze 1-6 hr after removal, were frozen in embedding medium (Tissue Tek II; Miles Laboratories) at -20°C; specimens in the second series were collected in culture medium and then snap frozen in embedding medium in isopentane cooled to ≤ -150°C in liquid nitrogen. Serial frozen sections on glass slides were hybridized as described.

RESULTS

In Situ Hybridization to Chicken Artery Wall. The 12 recombinant MDV plasmids, constituting approximately 40% of the MDV genome, were nick-translated with tritiated nucleotides for use in in situ hybridization. The ability of these probes to detect MDV mRNA was tested on MDV-transformed lymphoblastoid cell lines MDCC-CU36 and MDCC-CU29. The MDV transformed cells were strongly positive, similar to previously reported results (23). The same probe on chicken embryonic fibroblasts obtained from specific pathogen-free birds gave negative results, as did the EBV-cloned probe and HSV-2 probes on the MDV-transformed cell lines, showing that the hybridization is specific.

To pursue the question of persistence of MDV gene expression in atherosclerotic lesions of chicken artery wall, in situ hybridization was done with frozen sections prepared from arterial tissue collected both from chickens infected with the CU-2 strain of MDV (14) and from specific pathogen-free birds (generously provided by Catherine and Julius Fabricant). Serial sections were hybridized in situ with the MDV probe and, as a control, with a subgenomic clone of HSV-DNA (pDG201). The result of a preliminary experiment is shown in Fig. 1. Hy-

![Fig. 1. In situ hybridization of [³H]MDV DNA to a chicken atherosclerotic lesion. (A) Low-power view of a section of a gastric artery taken from a chicken infected with MDV. (Hematoxylin/eosin; bar = 100 μm.) (B) High-power view of a portion of the same vessel (indicated by the box) hybridized in situ with a mixture of 12 tritiated MDV plasmids. Arrows indicate positive cells. (Exposure, 8 wk; Giemsa; bar = 10 μm.)](image-url)
bridization of MDV DNA was restricted to cells within the thickened intima and was to some extent focally distributed within the plaque.

**In Situ Hybridization to Human Artery Specimens.** In the initial series, 160 anterior ascending thoracic aortic specimens from patients were subjected to *in situ* hybridization using the first of our two *in situ* hybridization protocols. Two probes were chosen, the full HSV genome and the pDG201 cloned fragment, the latter containing sequences that code for an early gene product in virus infection. This fragment is known to have some homology with mammalian DNA (26). Most of these specimens showed very poor histologic preservation during the *in situ* hybridization process and gave negative results. In addition, of those that were reasonably well preserved, few gave evidence of any significant intimal thickening and overt intimal lesions were rare. The positive results from this initial series fall into two categories. In one case, small intimal lesions contained cells that gave positive results with both the full HSV genome and pDG201. All of the cells constituting the lesion were positive with occasional additional positive cells scattered in the adjacent intima. Repeat hybridization, including a negative control with CMV, confirmed the presence of HSV mRNA in these cells. In 10 specimens, all of which showed normal histology, cells scattered throughout the medium were positive with HSV-2 but generally negative with pDG201, its subgenomic fragment.

In the second series, in which immediate freezing and fixation with 0.1% glutaraldehyde prior to hybridization was used, the tissue preservation was much improved. Four cases were selected out of a group of 17 for hybridization because they contained abnormally thickened intima. A battery of probes was used: the full HSV genome, subgenomic fragments, pDG201, pDG401, CMV, EBV, and MDV. Two of these four specimens were strongly positive with the full HSV genome; in addition, one was positive with the pDG201 and the other with the pDG401 probe. The thickened intima of one of these two positive specimens (Fig. 2) contained three discrete foci of increased cellularity in which all cells were positive; between these foci lay occasional scattered positive intimal cells, whereas the medial cells were negative. The other specimen had a discrete focus of strongly positive cells in the outer portion of the media, directly underlying the thickened intima. Within this focus, all cells gave positive results with both the full HSV genome and pDG401 and the normal histology of the media was disrupted: the focus was hypercellular compared with normal adjacent media and the connective tissue bands were thinned and discontinuous. All four of these specimens were negative for EBV, CMV, and MDV. In all positive specimens, the artery wall cells reacting with the HSV probes appear to be smooth muscle cells by location, morphology, and staining properties.

**Infection of Human Vascular Smooth Muscle Cells with Herpesvirus.** To answer the question of whether or not herpesvirus can infect human arterial smooth muscle cells, we proceeded as follows. Cultured human smooth muscle cells were exposed to virus and then examined both by *in situ* hybridization and for productive infection with HSV-1 by plaque assay. When cells were infected with one each of four herpesviruses cultured for various time periods and then assayed for the presence of viral mRNA by *in situ* hybridization, the following was observed. Both types of HSV produced classic cytopathic effects with cell retraction and rounding. *In situ* hybridization gave strongly positive results with HSV-1 and HSV-2 specimens fixed 6 and 8 hr, respectively, after infection and by 24 hr cytopathic effects were extensive. The effects of CMV and EBV were considerably different. CMV caused no evident cytopathic effects and the cells continued to proliferate in the pattern characteristic of smooth muscle cells for 48 hr. A control culture of mock-infected cells had the same growth pattern over this time period. *In situ* hybridization with the CMV probe gave negative results at 24 and 48 hr. EBV, on the other hand, caused an immediate change in the morphology of the cell culture, inducing multiple focal clusters of smooth muscle cells within the first hour of infection. These focal clusters persisted for the next 48 hr, with little evident change in overall cell number or morphologic pattern. *In situ* hybridization gave negative results at both 24 and 48 hr.

Plaque assay showed that HSV-1 is capable of proliferating in cultured human fetal aortic smooth muscle cells; a 100-fold increase in virus titer was observed at 48 hr.

**DISCUSSION**

In the preliminary observation with a mixture of probes representing 40% of the MDV genome, we found MDV mRNA in some cells of an atherosclerotic lesion of chicken artery. This result is of interest because application of fluorescent antibodies of MDV internal antigen revealed positive cells soon after infection, but no antigen was detected in mature plaques, although antigen-positive cells were seen around plaques (15). Together, these results suggest that virus or some part thereof may indeed persist and induce cell proliferation leading to lesion formation.

Our *in situ* hybridization results indicate that HSV mRNA...
(27–29) is present in medial and intimal cells of some aortic specimens taken from patients with clinically significant atherosclerosis. At the moment, we cannot be certain whether we are dealing with HSV-1 or HSV-2 because they have approximately 50% sequence homology (30) and all of the cloned HSV-2 probes used will hybridize with HSV-1 DNA. It is important to emphasize that certain of these specimens appear to represent early lesions in plaque development. At present, we are unable to draw any conclusions about the incidence of this occurrence for two reasons: (i) although the small number of positive results may seem to indicate a low incidence, many of the tissue samples were not optimally preserved to withstand the hybridization procedures and (ii) the region sampled, the anterior ascending thoracic aorta, is relatively free of atherosclerotic disease. The presence of HSV in two of a group of four specimens in our second series, with the improved collection and fixation procedures and the selection of tissues with intimal lesions, suggests that the incidence may be much higher.

The fact that both HSV-1 and HSV-2 are able to infect cultured human smooth muscle cells is corollary evidence supporting the in situ hybridization data. Others have shown that HSV-1 can cause productive infection in cultured endothelial cells (31) and that infectious virus may be released from HSV-1-infected fetal aortic explants for up to 20 days (32). Although such in vitro observations offer suggestions, they cannot directly inform about the in vivo behavior of HSV.

The virus having biological properties most similar to MDV is EBV (33); however, we have not so far found evidence of EBV nucleic acid associated with cells in arterial walls of arterial lesions. CMV, like the herpes simplex viruses, is widely distributed in humans and can remain latent indefinitely. Furthermore, these viruses may be transcribed in the latent state and the RNA transcripts can be detected by using in situ hybridization (27–29). In the small number of specimens we examined, CMV was not found. It should be noted that the presence of CMV has been detected by in situ hybridization in endothelial cells of small vessels in patients dying with widespread CMV infection after bone marrow transplant procedures (34).

Viruses and cells may have one of three general relationships: (i) permissive infection leading to virus release and cell lysis, (ii) a state of virus latency (e.g., within the cells comprising the lesion), or (iii) a relationship in which enhanced cell proliferation results either from the persistence and probable expression of part or all of the virus genome or from a virus-induced alteration similar to a mutation in the genome of the cell (35). A virus etiology is compatible with our current concepts of the importance of cell proliferation and either of the presumed mechanisms leading to such proliferation in atheromatous plaque formation. Furthermore, it could explain several hitherto puzzling features in the occurrence of atherosclerosis and thrombosis: (i) intimal cell proliferation in the absence of certain common risk factors, (ii) the clonal nature of cell populations found in many human atherosclerotic lesions, (iii) a large component of risk associated with genetic factors other than hypercholesterolemia, and (iv) the role of certain environmental factors in eliciting vascular occlusive disease. Our preliminary results encourage us to further explore herpesviruses and other viruses, along with agents that may modify their expression in the vascular system, as possible etiologic factors in the development of atherosclerosis.

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