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Envelope Glycoprotein of Avian Hemangioma Retrovirus Induces a Thrombogenic Surface on Human and Bovine Endothelial Cells

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Vascular endothelial cells are a target for blood-borne pathogens which may affect their integrity and thromboresistant properties. Here, we report that cultured bovine and human endothelial cells lose their thromboresistance following interaction with the avian hemangioma-inducing retrovirus. We show that the envelope (*env*) gene product, glycoprotein 85, is responsible for this effect, which appears soon after infection without viral replication or cell transformation. Induction of thrombogenicity is associated with a reduction in prostacyclin release and increased expression of tissue factor. These observations may explain the occurrence of thrombosis frequently observed in association with the hemangiosarcomas induced by avian hemangioma-inducing retrovirus. These unique endothelial cell-virus interactions may also be a model for the pathogenesis of various vascular diseases.

Endothelial cells (EC) provide a nonthrombogenic surface and act as a selective barrier between the blood and the vessel wall. Injury to the endothelium may promote thrombosis and, under certain conditions, cause disseminated intravascular coagulation or contribute to the formation of atheromatous lesions (4, 17). Certain bacteria may cause significant vascular damage either by invasion through the endothelium or by the deleterious effects of endotoxin (4, 14). Viruses may also affect the vascular endothelium. However, with the exception of the diseases caused by arboviruses (dengue and Junin viruses), which are associated with severe vascular damage manifested by hemorrhagic syndromes, other viruses cause only subtle perturbation of the EC (11, 12). Viral proteins, viral particles, and virus-related nucleic acids of measles virus, rubella virus, herpes simplex virus, cytomegalovirus, human immunodeficiency virus, and other viruses have been detected within EC and/or smooth muscle cells taken from artherosclerotic lesions (1, 3, 4). It has been proposed that subclinical viral infections which affect EC are among the inciting agents leading to the development of vessel disease (1, 3).

Infection of cultured EC with viruses often alters highly specialized endothelial functions such as adherence of granulocytes, production of colony-stimulating factors and interferon, synthesis of matrix proteins, induction of C3 and Fc receptors, and increased thrombogenicity (4, 7, 8, 15). All these effects appear to depend on viral replication or cellular transformation.

We have recently isolated a new field strain of avian leukosis virus from spontaneous hemangiosarcomas of layer hens. This newly isolated avian hemangioma retrovirus (AHV) induces tumors in about 30% of birds inoculated on the day of hatching (6). The resulting vascular tumors are composed of EC and small vessels and have a morphological similarity to Kaposi's sarcoma in acquired immune deficiency syndrome patients (D. Soffer, N. Resnick-Royal, A. Eldor, and M. Kotler, Cancer Res., in press).

We cloned AHV by DNA recombination techniques. Both the field isolate and the cloned virus induced cytopathic effects in cultured avian and mammalian cells (16). It could be shown that this effect is caused by the *env* product unique to AHV. UV-inactivated AHV, recombinants of Rous-associated virus (RAV) containing the AHV *env*, and partially purified AHV envelope glycoproteins were all cytopathic to avian and mammalian cultured cells (16). Unlike bacterial endotoxin, proteins of viral origin have not been known to cause EC perturbation. We report here the effects of AHV and its glycoproteins on certain specialized functions of endothelial cells in culture.

Bovine EC (BAEC) subconfluently plated on endothelial extracellular matrix (ECM)-coated dishes prevent ECM-induced platelet aggregation (10) (Fig. 1A). In this system, BAEC treated with AHV no longer prevented platelet aggregation 5 h posttreatment, prior to any apparent cell damage (Fig. 1C). This change in BAEC behavior is unique to AHV, since the exposure of BAEC to RAV type 1 (RAV-1), another member of the avian leukosis virus group whose genomic sequences are closely related to those of AHV, was not thrombogenic (Fig. 1B).

We constructed two recombinant viruses in order to determine whether AHV glycoproteins were responsible for the effect on BAEC. Treatment of EC with recombinant virus RxAHV1, a RAV-1 which contains the AHV *env*, caused platelet aggregation around the EC (Fig. 1E). This effect was not seen with AxHRV5, a recombinant virus expressing the RAV-1 *env* gene (Fig. 1F).

Since the effect of AHV on BAEC was observed as early as 5 h postinfection, we suggest that virus replication was not involved. This was confirmed by demonstrating that UV-inactivated AHV is still effective in abolishing the antiaggregating effect of EC (Fig. 1D). It is known that mammalian cells are not permissive for most avian sarcoma-leukemia viruses, none of which can propagate in those cells (2, 13). On the other hand, vesicular stomatitis virus (VSV), which replicates and destroys cultured BAEC, did not impair the capacity of EC to prevent platelet aggregation in the ECM system (Fig. 1G). It seems, therefore, that the effect of the AHV glycoproteins which impair the thrombo-

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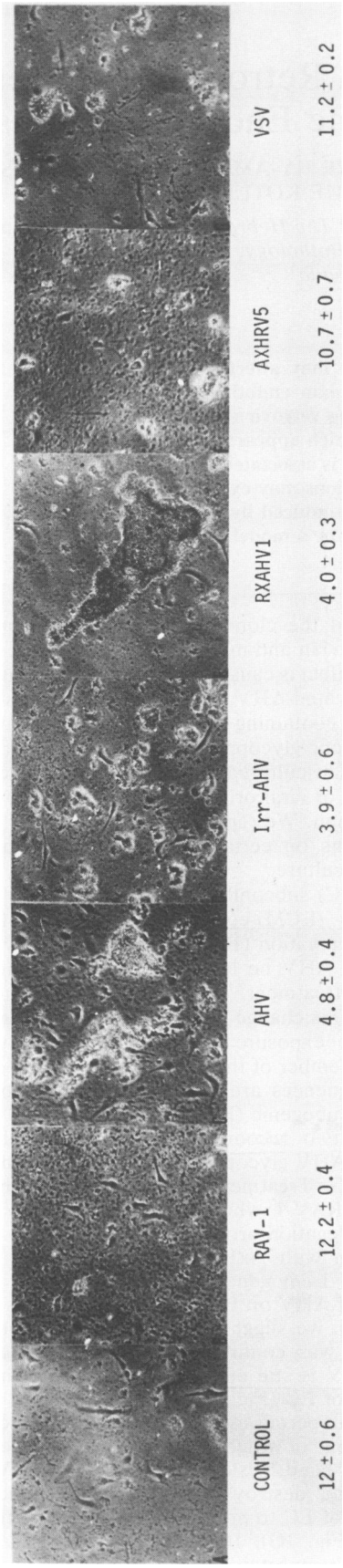

FIG. 1. Platelet aggregation around treated (B through G) and untreated (A) BAEC plated on ECM and the release of PGI_2 (nanograms per milliliter, ± standard deviation) from those cells (micrographs taken at 5 h postinfection). BAEC (7×10^4) were plated on 35-mm ECM-coated dishes. After 12 h, the cells were incubated for 1 h with 5 infectious units (multiplicity of infection, 5) of RAV-1 (B), AHV (C), irradiated AHV (D), RxAHV1 (E), AxHRV5 (F), or VSV (G) per cell. Viral titers were determined on chick embryonic fibroblasts by endpoint dilution and reverse transcriptase assay, as described before (16). Cultures were washed and incubated for an additional 4 h with fresh medium. The medium was then removed, the cells were incubated with platelet-rich plasma for 30 min, and platelet aggregation was observed with an inverted microscope, as previously described (10). The PGI_2 content in the supernatant harvested from each plate was determined after the platelets were removed as previously described (9).

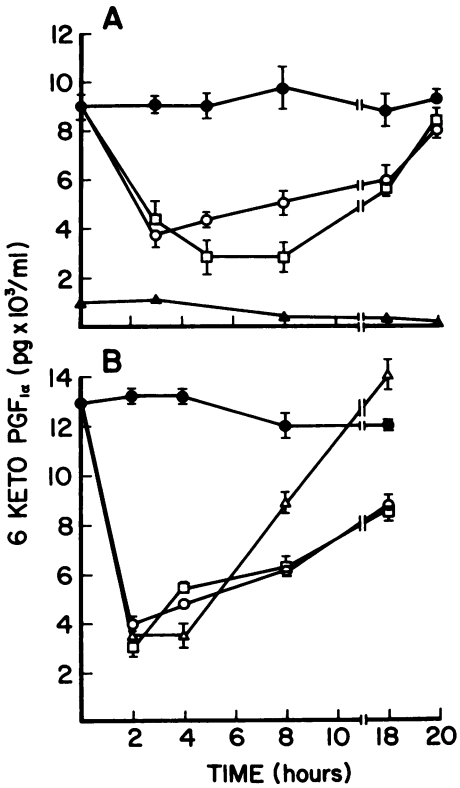

FIG. 2. Release of PGI_2 from treated and untreated EC. (A) BAEC (7×10^4 cells) plated on 35-mm ECM-coated plates and treated with viruses for various intervals, as described in the legend to Fig. 1. At the indicated times, the cells were washed twice and incubated with platelet-rich plasma for 30 min and the amount of PGI_2 released to the media was determined (9) for untreated (●), AHV-treated (○), and irradiated-AHV-treated (□) cells and ECM-coated plates without BAEC (▲). (B) Human umbilical vein EC (10^5) plated on 24-well plates, as described by Friedman et al. (12). Forty-eight hours later, the cells were incubated with the indicated viruses (multiplicity of infection, 5) or treated with LPS (1 μg/ml) for various times. At the end of each period, the cells were washed and incubated with fresh medium for 30 min and the amount of PGI_2 released to the media was determined for untreated (●), AHV-treated (○), SU (2 ng per well)-treated (□), and LPS (1 μg/ml)-treated (△) human umbilical vein EC. 6 Keto $PGF_{1\alpha}$ is the stable hydration product of PGI_2 measured by radioimmunoassay (9).

resistant properties of EC is unique and is not solely associated with cellular destruction or death.

As several viruses are known to induce platelet aggregation directly (7), we incubated platelets with AHV and recorded whether aggregation occurred in a Born-type aggregometer. AHV did not directly induce platelet aggregation, nor did it act synergistically with other platelet agonists to induce aggregation (results not shown). Such experiments exclude a direct effect of AHV on the platelets and confirm an AHV-induced EC change.

Inhibition of platelet aggregation in the ECM system is mediated by prostacyclin (PGI_2) secretion from the endothelium (10). Figure 1 shows that the occurrence of platelet aggregation adjacent to perturbed EC is associated with lower PGI_2 production. The rate of PGI_2 secretion by AHV-treated bovine and human EC was reduced two- to threefold in AHV-treated cells compared with untreated cells (Fig. 2). This inhibitory effect was evident as early as 3

h after viral treatment and decreased afterwards. A similar reduction in PGI_2 was also shown for EC treated with UV-inactivated virus or with partially purified glycoprotein 85 (SU). (Isolation of SU was carried out by mild treatment of purified virions with Triton X-100, followed by extraction with butanol, as previously described [16].) The observation (Fig. 2) that treated cells release higher amounts of PGI_2 into culture media 20 h posttreatment is explained by the progressive cytopathic effect of AHV on these cells (16). EC damage is associated with activation of the arachidonic cascade and the release of PGI_2 (18). The amount of PGI_2 secreted from VSV-infected BAEC was unchanged at 30, 60, and 180 min postinfection. However, beginning at 5 h postinfection, the cells started to release increased amounts of PGI_2. At 24 h, the amount released was fivefold higher than that of the uninfected cells. VSV-induced cell killing on these cultures started 6 h postinfection (data not shown).

It is of interest that the treatment of EC with lipopolysaccharide (LPS) is also associated with impaired release of PGI_2 during the first 4 h (Fig. 2). The RxAHV1 recombinant virus containing the AHV *env* gene was as inhibitory to PGI_2 secretion as AHV (Fig. 1), while incubation of bovine EC with RAV-1 or with the reciprocal recombinant virus AxHRV5 did not impair PGI_2 secretion. These results confirm the hypothesis that the AHV SU is responsible for the observed alterations in the capacity of EC to release PGI_2.

Perturbation of EC function can also be accompanied by the expression of membrane tissue factor (TF) activity. Quiescent EC do not express this factor, whereas its activity may be induced by molecules such as interleukin-1, LPS, and tumor necrosis factor. We determined, therefore, whether AHV SU also enhances TF expression by a method based on the production of procoagulant activity (5). The levels of TF expressed by BAEC treated with AHV and by BAEC treated with RxAHV1 recombinant virus were 60- and 140-fold higher, respectively, than that expressed by untreated cells. Human EC treated with partially purified AHV glycoproteins expressed about a 100-fold increase in TF. In contrast, treatment with RAV-1 or AxHRV5 recombinant virus did not increase TF. The induction of TF was rapid but transient, lasting less than 20 h after treatment. EC treated with VSV and LPS showed elevated amounts of TF which lasted only for 8 h (Fig. 3).

The effects of AHV and its glycoproteins on EC cultures are probably operative in the pathogenesis of AHV disease in chickens. The vascular tumors produced by AHV are associated with the presence of large thrombi formed around and within the lesions (Soffer et al., in press). These avian hemangiosarcomas are composed of EC whose surfaces have apparently become thrombogenic. Unfortunately, avian EC are as yet impossible to cultivate, so the direct effect of AHV and its glycoproteins on EC thromboresistance cannot be evaluated.

Hemangiosarcoma has been studied recently in two experimental models. (i) Human Kaposi's sarcoma tumor cells were transplanted into nude mice. This induced the formation of Kaposi-type tumors with the malignant cells of murine origin. The transplanted human cells disappeared after several days (20). Since in human Kaposi's sarcoma (frequent in some groups of acquired immune deficiency syndrome patients), no human immunodeficiency virus-genomic RNA, proviruses, or viral proteins are detected, it is not yet clear how such tumors were induced in the recipient mice. (ii) Transgenic mice carrying a retroviral vector expressing the polyomavirus middle-T antigen were found to develop hemangiomas at an early age. Cells cultured from

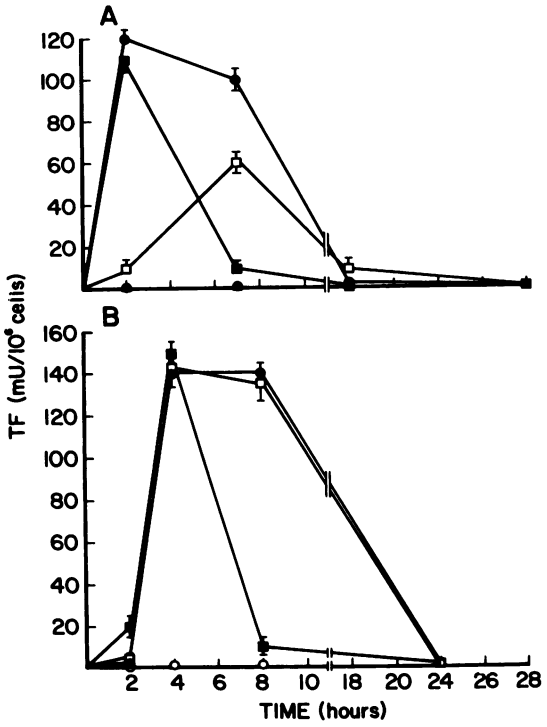

FIG. 3. Determination of TF activity on AHV-treated EC. BAEC (10^5) (A) and human umbilical vein EC (B) were seeded on 24-well plates. Four-day-old cultures were treated for 1 h with the viruses, washed, and then incubated for various times. At the end of each period, cells were scraped from the wells and transferred into buffer containing 0.1 M NaCl, 50 mM Tris hydrochloride (pH 7.5), and 1 mg of bovine serum albumin per ml. They were frozen and thawed twice and then assayed for TF activity (5) by incubating 0.1 ml of cell extracts with 0.1 ml of prewarmed human plasma and 0.1 ml of $CaCl_2$ (20 mM). A linear standard curve (log TF versus log clotting time) was used to quantify TF activity. One unit of TF was defined as the concentration of extracted rabbit brain clotting within 25 s. Each experiment was performed in triplicate. (A) Untreated (○), RAV-treated (△), and AxHRV5-treated (▲) BAEC (all located on the base line) and AHV-treated (●), RxAHV1-treated (□), and VSV-infected (■) BAEC. (B) Untreated (○), AHV-treated (●), SU (2 ng per well)-treated (□), and LPS (1 μg/ml)-treated (■) human umbilical vein EC.

these tumors induced hemangiosarcoma by recruiting cells of the recipients as early as 1 to 3 days posttransplantation (19). It seems likely that the initiating agents in both models stimulate the production and secretion of growth factors or chemotactic factors responsible for tumor formation or both.

We show here that AHV glycoprotein perturbs EC, causing reduced secretion of PGI_2, increased expression of TF activity, and high expression of interleukin-1 (Resnick-Roguel et al., unpublished results). It is clear from this study that the EC perturbation, as well as the cytopathic effects, is caused by the AHV envelope glycoprotein. These glycoproteins may also play a role in the induction of hemangioma and other endothelium-related vascular diseases.

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