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Identification of a monocyte receptor on herpesvirus-infected endothelial cells

(cell adhesion molecule GMP140/herpes simplex virus 1/atherosclerosis/inflammation)

ORLI R. ETINGIN*, ROY L. SILVERSTEIN*, AND DAVID P. HAJJAR†

Departments of *Medicine, †Biochemistry and Pathology, and the National Institutes of Health Specialized Center for Thrombosis Research, Cornell University Medical College, New York, NY 10021

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ABSTRACT **The adhesion of circulating blood cells to vascular endothelium may be an initial step in atherosclerosis, inflammation, and wound healing. One mechanism for promoting cell–cell adhesion involves the expression of adhesion molecules on the surface of the target cell. Herpes simplex virus infection of endothelium induces arterial injury and has been implicated in the development of human atherosclerosis. We now demonstrate that HSV-infected endothelial cells express the adhesion molecule GMP140 and that this requires cell surface expression of HSV glycoprotein C and local thrombin generation. Monocyte adhesion to HSV-infected endothelial cells was completely inhibited by anti-GMP140 antibodies but not by antibodies to other adhesion molecules such as VCAM and ELAM-1. The induction of GMP140 expression on HSV-infected endothelium may be an important pathophysiological mechanism in virus-induced cell injury and inflammation.**

Vascular endothelial injury is a common inciting factor in the molecular pathways leading to inflammation, thrombosis, and atherogenesis. Such an injury can be caused by mechanical, chemical, or viral pathogens, particularly the enveloped DNA viruses such as herpes simplex virus (HSV) or cytomegalovirus (1, 2). The role of DNA viruses in the pathogenesis of atherosclerosis is suggested by the observation of HSV genomic material in human atherosclerotic lesions and by biochemical and histologic alterations in vascular cells caused by avian herpesvirus (Marek disease) infection that are virtually indistinguishable from human atherosclerosis (3–5). *In vitro* effects of HSV infection of human vascular cells parallel those seen in the development of the human atheromatous lesion—namely, accumulation of cholesteryl esters, increased expression of tissue factor, and adhesion of inflammatory cells (6–9).

Adhesion of circulating leukocytes to injured endothelium is an important early step in the development of the atherosclerotic lesion. Studies have identified monocyte-derived growth factors, cytokines, and other leukocyte products in the infected endothelium. HSV-infected endothelium, like endothelium exposed to tumor necrosis factor or interleukin 1, binds platelets and leukocytes more avidly than noninfected cells (8–10). We have recently shown (11) that human monocytes and cells of the monocyte-like cell line U937 preferentially adhere to HSV-infected endothelial cells and that this adhesion requires both localized thrombin generation and surface expression of HSV-encoded glycoprotein C (gC).

HSV-infected cells express several transmembrane glycoproteins encoded by the HSV genome which are involved in molecular mimicry that may play a role in the pathogenesis of injury. Glycoprotein E (gE), for example, can act as an Fc receptor, while gC can mimic at the molecular level a complement (C3b) receptor (12, 13). HSV gC expressed on infected endothelial cells binds and activates factor X, thereby promoting thrombin generation on the endothelial surface (11). A role for these proteins in the pathogenesis of injury is further suggested by the observation that adhesion of neutrophils to HSV-infected endothelium was blocked by a nonspecific anti-viral serum (7).

Endothelial cells express several leukocyte receptors, including GMP140 (also known as PADGEM or CD62), ELAM-1, ICAM-1 and -2, and VCAM-1, on their surfaces in response to cytokines or other agonists (14–19). We now show that HSV-infected human endothelial cells express the adhesion molecule GMP140. Expression of this protein was responsible for enhanced monocyte adhesion to the infected cells and required localized thrombin generation and surface expression of viral gC.

MATERIALS AND METHODS

Cell Culture. Human umbilical vein endothelial cells (HUVECs) and the cells of the monocytoid line U937 were grown as described (11). HUVECs were subcultured one to three times prior to use and were confirmed to be endothelial cells by immunofluorescent staining with antiserum to von Willebrand factor (20). All HUVECs in an individual experiment were obtained from the same umbilical cord. Human peripheral blood monocytes were isolated by successive centrifugation on Ficoll/Hypaque and Sepracell-MN (Sepratech, Oklahoma City, OK) (11).

Virus. HSV-1 (strain F) was purchased from the American Type Culture Collection. Confluent monolayers were infected with 1.0 plaque-forming unit of HSV per cell as described (11). In some studies, HUVECs were also infected separately with previously characterized HSV-1 mutants: MP^-, which does not express gC, or NS-1 or gC551, which produce a truncated gC not expressed on the HUVEC surface (11).

Antibodies. Murine monoclonal antibodies to GMP140 were provided by R. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK) (antibody G1) and B. Furie and A. Celi (Tufts University, Boston) (AC1.2). Rabbit anti-GMP140 serum was also provided by B. Furie. Murine monoclonal anti-ELAM-1 (H18/7) and anti-VCAM (E16) hybridoma culture media were obtained from M. Bevilacqua (Brigham and Women's Hospital, Boston), and anti-gC from H. Friedman (University of Pennsylvania, Philadelphia). Fluorescein-conjugated goat anti-mouse IgG was purchased from Tago.

Endothelial Cell–Monocyte Adhesion Assay. Monocyte adhesion to HUVECs was assayed using ^{51}Cr-labeled U937 monocytic cells (11). After incubation of ^{51}Cr-labeled U937

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Abbreviations: HSV, herpes simplex virus; gC, glycoprotein C; gE, glycoprotein E; HUVEC, human umbilical vein endothelial cell; HBSS, Hanks' balanced salts solution.

cells with HUVECs, nonadherent cells were gently removed by rinsing wells three times with 0.5 ml of phosphate-buffered saline. Cell monolayers were solubilized in 1.0-ml aliquots of 0.2 M NaOH. Hirudin (0.5 mg/ml, Sigma) or D-Phe-L-Pro-L-Arg-CH_2Cl ("D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone," PPACK), inhibitors of thrombin, was added to certain wells 1 hr before addition of monocytes or ^{125}I-labeled anti-GMP140. In some studies, U937 cells were fixed prior to addition to endothelial cell monolayers. For fixation, U937 cells labeled with ^{51}Cr were incubated at 4°C for 1 hr in 1% paraformaldehyde in Hanks' balanced salts solution (HBSS) buffered to pH 7.4 with 20 mM Hepes. Cells were washed three times in HBSS/Hepes, centrifuged at 600 × *g* for 10 min and resuspended in 1:1 RPMI 1640 with 20% fetal bovine serum/medium 199 prior to the addition to confluent HUVEC monolayers.

^{125}I-anti-GMP140 Binding Assay. Cell surface GMP140 expression was quantitated by binding of ^{125}I-conjugated anti-GMP140 IgG (AC1.2). ^{125}I-labeled antibody (2 μg/ml, 1.14×10^4 μCi/mol; 1 Ci = 37 GBq) was added to confluent endothelial cell monolayers in a 24-well plate (Costar) containing 150 μl of HBSS/1% bovine serum albumin per well. After incubation at 22°C for 1 hr, the supernatants were removed. The cells were washed three times in HBSS and then lysed in 250 μl of 0.2 M NaOH for isotope counting. Nonspecific binding was measured in the presence of 100-fold excess unlabeled IgG and did not exceed 10%.

Flow Cytometry. HUVECs were mock-infected or infected with HSV as described above 18–24 hr prior to cytometric analysis. After infection, cells were scraped from the culture dishes, washed three times in HBSS, and resuspended in phosphate-buffered saline containing 1% goat serum, 0.3% bovine serum albumin, and 0.02% NaN_3. Cells were then incubated with murine monoclonal anti-GMP140 AC1.2 ascites or control nonimmune ascites, both at 1:250 dilution, for 1 hr at 4°C. In separate wells, cells were similarly incubated with anti-gC at 1:250 dilution. The cells were then washed three times and resuspended with fluorescein-conjugated goat anti-mouse IgG at a 1:100 dilution in the same buffer. After 1 hr at 4°C, the cells were washed three times, resuspended in filtered phosphate-buffered saline, and analyzed with an Epics flow cytometer (Coulter) equipped with an MDADS data handling computer and an argon laser. Sizing gates were set to include all nucleated cells. At least 10^4 cells were analyzed for each sample.

Statistics. All data were analyzed by analysis of variance.

RESULTS

HSV-Infected Endothelial Cells Express GMP140. Indirect immunofluorescence flow cytometry with specific monoclonal antibodies was used to demonstrate that HSV-infected HUVECs expressed GMP140 18–24 hr postinfection (Fig. 1 *Upper*). More than 70% of the infected cells bound anti-GMP140, compared with less than 10% of the mock-infected cells. Viral gC was expressed on 90% of HSV-infected cells at the same time point, whereas no gC was detected on mock-infected cells (Fig. 1 *Lower*). Isotype-matched control nonimmune antibodies and anti-ELAM-1 did not bind to the HSV-infected cells (data not shown).

GMP140 Mediates Monocyte Adhesion to HSV-Infected Endothelial Cells. We previously showed that HSV-infected endothelial cells bound 2- to 4-fold more U937 monocytic cells and peripheral blood monocytes than mock-infected or adenovirus-infected cells (11). We now show that monoclonal and polyclonal antibodies to GMP140 abolished this adhesion (Fig. 2). Neither a monoclonal antibody directed against ELAM-1, nor nonimmune murine IgG, nor rabbit nonimmune serum blocked monocyte adhesion. Similar results were observed with freshly isolated human peripheral blood monocytes. Furthermore, antibodies to other adhesion molecules, including glycoprotein IV (CD36), VCAM-1, throm-

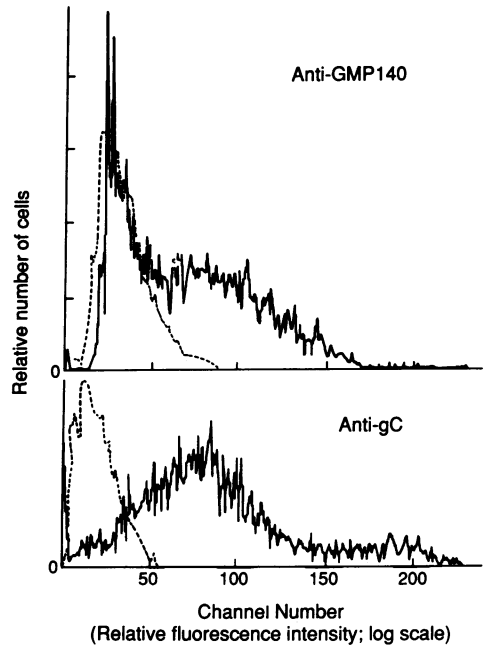

FIG. 1. HSV-infected HUVECs express GMP140. HUVECs were infected with 1.0 plaque-forming unit of HSV per cell and examined 18–24 hr later. Cells were processed for indirect immunofluorescence with anti-GMP140 AC1.2 ascites (1:250 dilution) or anti-gC (1:250) as described in *Materials and Methods*. After washing and resuspension, cells were analyzed by flow cytometry with sizing gates set to include all nucleated cells. For each sample at least 10^4 cells were analyzed. (*Upper*) Fluorescence with anti-GMP140, comparing HSV-infected cells (solid line) with mock-infected cells (broken line). (*Lower*) Fluorescence with anti-gC, comparing HSV-infected cells (solid line) with mock-infected cells (broken line).

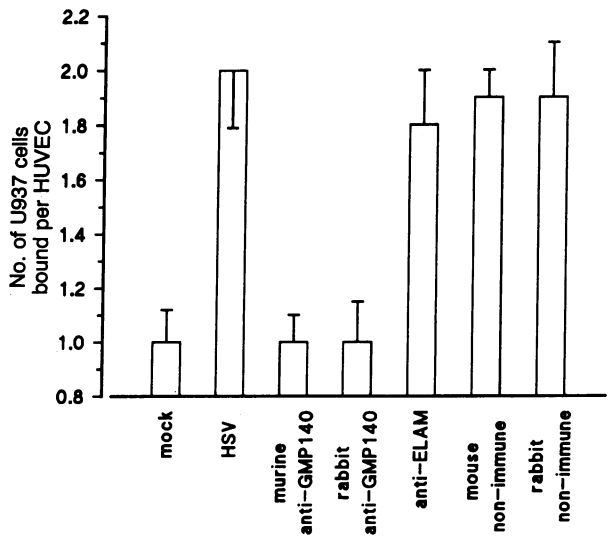

FIG. 2. Antibodies to GMP140 inhibit U937 cell adhesion to HSV-infected HUVECs. Confluent HUVECs infected with HSV were incubated with ^{51}Cr-labeled U937 cells at 4°C for 2 hr (11). After incubation, nonadherent cells were gently removed by rinsing, and the adherent cells were quantified by scintillation counting after solubilization in 0.2 M NaOH. Murine monoclonal anti-GMP140 IgG (antibody G1, 10 μg/ml) was added to infected endothelial cells 30 min before the addition of monocytes, as was nonimmune murine IgG, rabbit anti-GMP140 serum (1:100), nonimmune rabbit serum, and monoclonal anti-ELAM-1. Results are expressed as mean ± SD from three separate experiments each of which was performed in quadruplicate.

bospondin, and the leukocyte integrin CD11/18, did not inhibit monocyte adhesion to HSV-infected cells (data not shown). Similarly, the tetrapeptide Arg-Gly-Asp-Ser (RGDS) had no effect on monocyte adhesion to infected cells, and monocytes fixed in paraformaldehyde adhered to infected cells to a similar degree as fresh monocytes. In time-course studies we found that enhanced monocyte adhesion to infected endothelial cells was seen as early as 2 hr after infection, peaked at ≈4 hr, and persisted for at least 24 hr (Fig. 3*A*). GMP140 expression, as measured by binding of ^{125}I-labeled monoclonal antibody, closely paralleled monocyte adhesion (Fig. 3*B*). The enhanced adhesion was completely blocked by anti-GMP140 IgG at all time points observed, suggesting that GMP140 is the predominant monocyte receptor on HUVECs. The addition of anti-ELAM-1 antibodies or irrelevant antibodies did not inhibit monocyte adhesion to HSV-infected endothelial cells at any time point (data not shown).

GMP140 Expression Requires gC and Thrombin. Previous work showed that monocyte adhesion to HSV-infected endothelial cells was mediated by localized thrombin generation. This process required surface expression of HSV gC, which functioned as a factor X binding and activation site (11). To test the hypothesis that GMP140 expression on HSV-infected endothelial cells is similarly dependent on localized thrombin generation, we examined binding of ^{125}I-anti-GMP140 to confluent layers of endothelial cells infected with HSV. The infected cells bound significant amounts of ^{125}I-anti-GMP140 (110 pmol/cm^2; Fig. 4). This level of expression was similar to that induced by thrombin stimulation (2 units/ml for 30 min) of noninfected cells. Similarly, exogenous thrombin augmented GMP140 expression as measured by ^{125}I-anti-GMP140 binding on cells at 4 hr but not at 24 hr after infection (data not shown). Endothelial cell expression of GMP140 was blocked when cells were treated with a specific inhibitor of thrombin [D-Phe-L-Pro-L-Arg-CH_2Cl (10 μM) or hirudin (0.5 mg/ml)] at times from 2 to 24 hr after infection. Endothelial cells did not express GMP140 when infected with HSV mutants that lack functional gC expression or when monoclonal anti-gC IgG was added to HSV-infected cultures (Fig. 4). These data suggest that gC-dependent thrombin generation is required for GMP140 expression on HSV-infected endothelial cells.

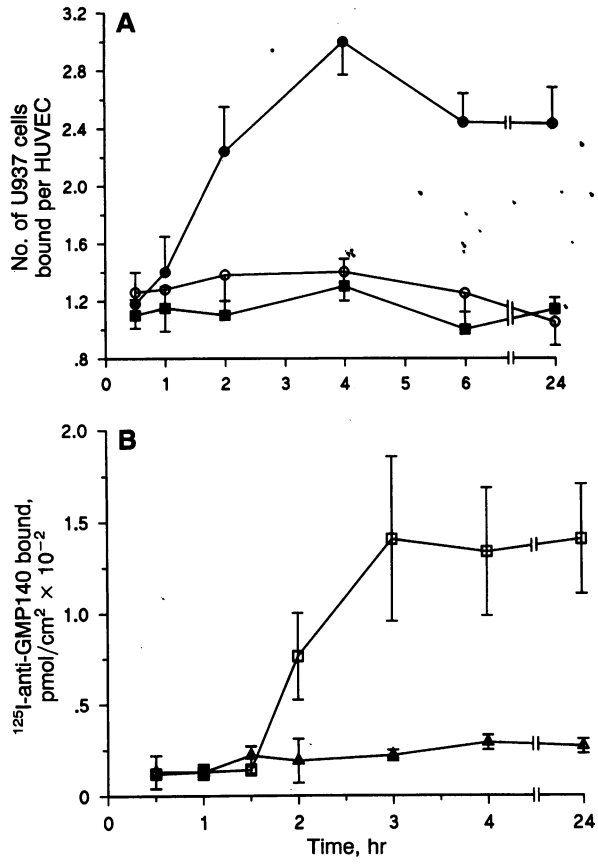

FIG. 3. (*A*) Time course of U937 monocyte adhesion to HSV-infected HUVECs. Mock-infected or HSV-infected HUVECs were washed with phosphate-buffered saline and refed with medium (RPMI 1640 with 20% fetal bovine serum/medium 199, 1:1). Monocyte adhesion was assayed at time points from 0.5 to 24 hr after removal of virus. Monocytes were prepared as described and stored at 37°C for up to 6 hr. Monoclonal anti-GMP140 IgG (○) or control IgG (●) were added to HSV-infected HUVECs 30 min before addition of monocytes as described in Fig. 2. Adhesion to mock-infected cells is also shown (■). Each point represents the mean ± SD for at least eight separate determinations. (*B*) Time course of GMP140 expression on HSV-infected HUVECs. Surface expression of GMP140 by mock-infected (△) or HSV-infected (□) HUVECs was quantitated using ^{125}I-conjugated anti-GMP140 IgG (AC1.2) at time points from 1 to 24 hr after infection. Results represent means ± SD from two experiments each of which was performed in quadriplicate.

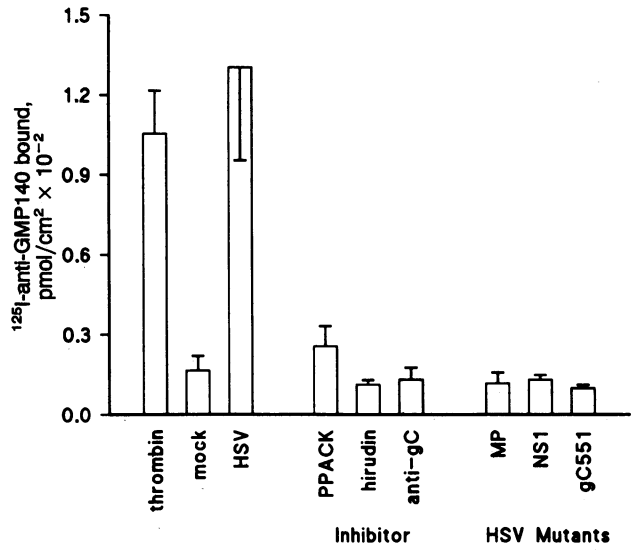

FIG. 4. GMP140 expression requires HSV gC expression and thrombin generation. Confluent HUVEC monolayers were washed with PBS and refed with medium 199. Cell surface GMP140 expression was quantitated using ^{125}I-conjugated anti-GMP140 IgG (AC1.2) 24 hr after infection. Hirudin (0.5 mg/ml) or D-Phe-L-Pro-L-Arg-CH_2Cl (PPACK, 10 μM) was added to certain wells 1 hr prior to addition of ^{125}I-anti-GMP140. In some studies, the cells were infected with previously characterized HSV-1 mutants: MP, which does not express gC, or NS-1 or gC551, which produce a truncated gC not expressed on the cell surface (11). Nonspecific binding was measured in the presence of 100-fold excess unlabeled IgG and did not exceed 10%. Results represent means ± SD from three separate experiments each performed in triplicate.

DISCUSSION

GMP140 is a cytoplasmic protein in resting endothelial cells that is found on the membrane of an intracellular organelle known as the Weibel–Palade body (14–19). After stimulation by thrombin, histamine, or complement proteins, the Weibel–Palade body is rapidly secreted and its membrane becomes incorporated into the plasma membrane, causing surface expression of GMP140 (15). This mechanism of new protein expression—i.e., translocation from a preformed intracellular membrane compartment to the cell surface—does not require *de novo* synthesis of GMP140 and is consistent with the inhibition of protein synthesis seen in HSV-

infected cells. Our data support the following model: HSV infection induces endothelial cell surface expression of HSV gC, which acts as a binding site for factor X. The concomitant generation of tissue factor converts bound factor X to an active prothrombinase leading to the generation of thrombin in the microenvironment of the infection where it can act in an autocrine manner to induce expression of the leukocyte receptor GMP140.

Several other potential leukocyte receptors are expressed on HSV-infected endothelial cells. HSV gE functions as an Fc receptor on infected endothelial cells and may utilize circulating IgG as an intercellular bridge between the infected cell and the circulating adherent granulocyte (12). Other examples of molecular mimicry include HSV gC, which functions as a C3b receptor and has been shown to mediate the adhesion of circulating complement-coated erythrocytes to infected endothelial cells (13). Our data show that the increase in monocyte adhesion induced by HSV infection can be blocked by anti-GMP140, suggesting that other receptors do not play a major role in this cell system. The leukocyte integrins (LFA-1, Mac-1, and p150,95) mediate adhesion to ICAM-1 and ICAM-2 on cytokine-stimulated endothelial cells (21) but do not appear to play a role in adhesion to the virus-infected cells, based on lack of inhibition seen with specific monoclonal antibodies, RGDS peptide, or monocyte fixation. Similarly, VCAM-1, which mediates lymphocyte adhesion to stimulated endothelial cells (22), does not appear to play a role, based on lack of inhibition seen with anti-VCAM antibodies. The leukocyte ligand for GMP140 has recently been characterized as a fucosylated carbohydrate similar or identical to the Lewis X antigen and CD15 (23).

The significance of a procoagulant phenotype of HSV-infected cells is several-fold. The local generation of thrombin at the site of infection may activate platelets as well as endothelial cells. Thrombin-stimulated platelets adhere to monocytes, and thus platelets may be recruited into the area of injury (24). The presence of activated platelets and monocytes at the area of infection may further contribute to the development of vascular injury and chronic inflammation (25). Expression of an adhesion molecule on HSV-infected cells may thus be an initial step in virally mediated endothelial injury. There is substantial evidence to suggest a potentiating role for HSV in the development of human atheroma. Benditt *et al.* (2) have reported that genomic material of HSV can be found in human atheroma (2), and avian herpesvirus infection causes a histologic picture and biochemical profile indistinguishable from human atherosclerosis (3, 5).

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