Original Article

MMP Cellular Responses to Dengue Virus Infection-Induced Vascular Leakage

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SUMMARY: Dengue hemorrhagic fever and dengue shock syndrome, the major life-threatening outcomes of severe dengue disease, are the consequence of plasma leakage in the vascular areas. We previously demonstrated that dengue virus (DV)-infected dendritic cells (DC) trigger vascular leakage through matrix metalloproteinase (MMP)-9 overproduction, however little is known concerning the consequences of direct infection of macrovascular endothelial cells (MVEC) by DV. In this study, we show that infection of primary human MVEC results in overproduction of MMP-2 and to a lesser extent of MMP-9, leading to enhanced endothelial permeability. This permeability was associated with loss of expression of the vascular endothelium-cadherin cell-cell adhesion. The MMP response to DV infection is strikingly different between DC and MVEC. Therefore, our results demonstrated that endothelial cells are an important target for DV infection, and that DV-induced MMP-2 overproduction by direct infection of endothelial cells may contribute to the pathogenesis of severe dengue infection.

INTRODUCTION

Dengue virus (DV) infection is amongst the most important emerging viral diseases transmitted by mosquitoes to humans, in terms of both illness and death (2). The worldwide large-scale reappearance of dengue over the past few decades has turned this disease into a major public health problem, especially in the tropical and subtropical regions (3).

DVs most commonly cause a benign febrile illness called dengue fever (DF) and less frequently cause a life-threatening illness characterized by disorder in hemostasis, endothelial permeability, thrombocytopenia, and coagulation (dengue hemorrhagic fever, DHF) (2,4). Vascular leakage is the hallmark of DHF that implies damage to the vascular endothelium, which constitutes the major permeability barrier in the vessel walls (5). Endothelial permeability alteration can lead to hypovolemic shock, a clinical situation called dengue shock syndrome (DSS) (6). The hypothesis, based on epidemiological data (7), that “DHF/DSS depends on a second heterotypic antibody-dependent enhancement (ADE) by DV infection” has not been validated, even if ADE has been shown to enhance the viral load (8). Results from retrospective analyses demonstrate that DHF/DSS can be induced after a primary infection (9,10), suggesting that DHF/DSS could depend on a combination of viral load, strain virulence and host immune response. It is widely believed that vascular leakage is caused by soluble mediators of inflammation (11,12). Increased serum levels of many circulating vasoactive factors, including cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, IL-8, interferon (IFN)-γ and IL-2 have been reported in DV-infected patients (13-17). Cells which support viral replication in vitro include dendritic cells (DCs) (18,19), macrophages (20), activated B-cells (21) and endothelial cells (22). It has been shown that cultured human endothelial cells were activated, as demonstrated by up-regulation of adhesion molecules, when exposed to culture fluids from dengue-infected peripheral blood monocytes (23). In a previous study, we clearly demonstrated that DV-infected immature DCs overproduce soluble matrix metalloproteinase (MMP)-9, which enhances endothelial permeability and could contribute to the molecular basis for DHF/DSS (1). Understanding DHF/DSS pathogenesis is of major importance due to the lack of a vaccine or effective treatment against DV. However, the molecular mechanisms associated with the increase of endothelial permeability due to direct infection of endothelial cells remain to be elucidated. Taking into account the fact that endothelial cells can be infected by DV (24-26) and that such infection alters cytokine production and modifies the expression of adhesion molecules on endothelial cells (12,26,27), we examined whether or not the infection of primary endothelial cells by DV might be involved in the pathogenesis of DHF/DSS through the production of MMP. Our results showed that DV infection could induce overexpression of MMP-2 in infected macrovascular endothelial cells (MVEC), which enhances endothelial permeability.

MATERIALS AND METHODS

Cells and DV: Primary human umbilical vein endothelial cells (MVEC) were cultured following a previously described method (28). The parental DV strain, 16681, pertaining to the DV2 subtype, was propagated in LLC-MK2 cells, and the virus titers expressed as plaque forming units (pfu) were determined by plaque assay, as described previously (29).

DV infection of MVEC and cell-stimulation conditions: A monolayer of 500,000 MVEC was exposed to DV for 2 h, at a multiplicity of infection (MOI) of 1 pfu/cell, washed twice to remove excess virus, and further cultured at 37°C and 5% CO2 in RPMI-1640, without fetal calf serum (FCS). The cells and culture supernatants used in subsequent experiments were collected in a kinetic way. In some experiments, after washing, cells were incubated in the presence of the p38 MAP-kinase inhibitor SB 203580 (Calbiochem, La Jolla, Calif., USA) or the MMP-inhibitor SB-3CT (Chemicon International,

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Detection of MMP activity: The gelatinase activity of MMP-2 or MMP-9 in cell culture supernatants from DV-infected and control MVEC was determined by SDS-PAGE zymography using a gelatin substrate, as previously described (30). Briefly, 500,000 cell culture supernatant was suspended in loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 0.04% bromophenol blue) and, without prior denaturation, was run on a 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin. Gels were subsequently stained with Coomassie brilliant blue G-250 and destained in 30% methanol/10% acetic acid (vol/vol) to detect gelatinase activity.

Protein arrays: Protein levels in the cell supernatants were analyzed by using the highly sensitive Searchlight™ Protein array technology (Pierce Endogen, Perbio, Boston, Mass., USA), based on protein detection by chemiluminescence.

Permeability assay: The permeability of the MVEC monolayer cultured on collagen-coated semipermeable membranes was assessed using a commercialized in vitro vascular permeability assay (Chemicon International), according to the manufacturer’s instructions. The amount of FITC-Dextran that permeated the MVEC monolayer into the plate well was determined by measuring the fluorescence at an excitation wavelength of 485 nm and emission at 530 nm in a spectrofluorometer (GENios-TECAN, Trappes, France).

Immunofluorescence microscopy: MVEC monolayers were exposed to DV for 2 h, at an MOI of 1 pfu/cell, washed twice to remove excess virus, and further cultured at 37°C and 5% CO2 in RPMI-1640, without FCS for 24 h. Following this incubation, MVEC were treated and incubated with the appropriate fluorescent staining system to observe the localization of either vascular endothelium (VE)-cadherin or nuclei under an inverted fluorescence microscope (Carl Zeiss, Gottingen, Germany).

RESULTS AND DISCUSSION

DV infection of human primary endothelial cells triggers the overproduction of MMP-2 and IL-8: Primary endothelial cells (MVEC) were exposed to DV, and the metalloproteinase activity in culture supernatants from infected and uninfected cells was analyzed by gelatin zymography at different post-infection times. Uninfected MVEC constitutively secreted basal levels of activated MMP-2 (72 kDa gelatinase) in a time-dependent manner (Fig. 1A). Although very low levels of MMP-9 (92 kDa gelatinase) activity were detected, MMP-2 activation was strongly enhanced following infection with DV (Fig. 1A). We also provide proof that the up-regulation of activated MMP-2 is p38-MAP-kinase activation-dependent (Fig. 1B). This figure also shows that SB203580 could be a potential inhibitor of activated MMP-2.

The amounts of MMPs and soluble factors associated with vascular leakage present in DV-infected MVEC were determined using chemiluminescence protein arrays. These results showed that DV-infection of endothelial cells strongly increased the production of the proinflammatory cytokines IL-8 and MMP-2, but not TNF-α, MMP-9 or MMP-13 (collagenase 3), as compared to uninfected MVEC (Fig. 2A-E). Interestingly, these data are reversed compared to those obtained upon DC-DV infection, suggesting that a synergistic effect of MMPs could locally destroy the basal membrane of blood vessels and cause hemorrhage. In addition, it has been reported that plasma and pleural fluids of patients suffering from DHF/DSS had a remarkably high level of IL-8 (24). The lower amount of MMP-13 secretion is in line with the observation that MMP-13 activates MMP-9 (31).

DV-infection of MVEC also induces the production of TIMP-1 and TIMP-2 (Fig. 2F, G), indicating that the enhanced production of the natural inhibitors of MMP-9 and MMP-2, respectively (32), was not able to restore the physiological balance between the MMP and TIMP and to block DV-induced vascular permeability.

DV-infected MVEC increase endothelial cell permeability in a MMP-2-dependent manner: As MMP-2 was overexpressed in the supernatants of DV-infected endothelial cells, we investigated whether direct infection of MVEC by DV could modify the permeability of endothelial cells. To this end, we used primary MVEC monolayers (33), which were exposed to DV for 2 h or left unexposed. After 24 h of post-infection, the soluble factors resulting from the interaction between cells and DV increased the permeability of the MVEC by about 3-fold, as compared to those from uninfected cells (Fig. 3). To establish whether the endothelium-permeabilizing activity of soluble factors was mediated by MMPs, the experiments were repeated in the presence of SB-3CT, a small and highly selective molecule that, at nanomolar concentrations, inhibits MMP-9 and MMP-2 gelatinase activities by directly binding to the catalytic zinc ion of these proteases (34). As shown in Fig. 3, SB-3CT restored the endothelial permeability of MVEC to basal levels, similar to those observed upon incubation with uninfected macrovascular cells. These results show that the increase in endothelial permeability is specifically mediated by MMP gelatinases that are secreted.
following infection of MVEC with DV. MMP-2 gelatinase seems to be the major factor involved in the endothelial permeability induced upon infection of macrovascular cells by DV.

**DV-infected MVEC induce MMP-2-mediated disruption of endothelial cell-cell adhesion:** Endothelial cells express several adhesion molecules such as cadherins that are broad-function adhesion molecules, concentrated in the areas of cell-cell contact, involved in changes of cell shape, as well as in endothelial cell junction activities (35). To visualize modifications of the integrity of endothelial junctions triggered by direct infection of MVEC by DV, we analyzed the distribution of VE-cadherin, in confluent MVEC exposed or not exposed to DV (Fig. 4). Results from these experiments showed that the expression of VE-cadherin was strongly decreased in MVEC monolayers cultured in the presence of DV, resulting in a significantly reduced cell-cell adhesion (Fig. 4A). Importantly, the observed disruption of cell-cell adhesion induced by DV-infected macrovascular cells was found to be strictly dependent on the activity of MMP-2 gelatinase, as the addition of SB-3CT prevented the disruption of endothelial cell-cell adhesion molecules (Fig. 4C).

Taken together, these findings provide support for a scenario in which increased vascular permeability leading to vascular leakage in MVEC dengue viral infection is directly linked to MMP-2 overproduction. It is possible that MMP-2 induced by DV infection of endothelial cells may have a severe local effect in this specific tissue. Our future studies will focus on the quantification of MMP-2 and MMP-9 in the sera from dengue patients at different stages of the disease. In conclusion, this is the first study showing the pathophysiologic significance of MVEC and MMP-2 in dengue without any significant production of MMP-9.

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