MCP-1, a highly expressed chemokine in dengue haemorrhagic fever/dengue shock syndrome patients, may cause permeability change, possibly through reduced tight junctions of vascular endothelium cells

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Received 3 April 2006
Accepted 7 August 2006

Vascular leakage, one hallmark of dengue haemorrhagic fever (DHF) and dengue shock syndrome, has been linked to the mediators secreted from cells in the circulatory system. In this study, extremely high expression levels of monocyte chemoattractant protein-1 (MCP-1) were found in the plasma of DHF patients compared with low MCP-1 expression levels in the plasma of enterovirus 71-infected patients. It was also found that MCP-1 expression was induced in dengue virus 2 (DV2)-infected monocytes and lymphocytes, but not in liver or endothelial cells. Exposing monolayers of human umbilical vein endothelial cells (HUVECs) to recombinant human MCP-1 (rhMCP-1) or to the culture supernatant of DV2-infected human monocytes increased the vascular permeability of the cells. MCP-1-neutralizing monoclonal antibody only partially prevented monolayer permeability change. Consistently, the distribution of the tight junction protein ZO-1 on the cellular membranes of HUVECs was disrupted by rhMCP-1 or by the conditioned medium of DV2-infected monocytes. In summary, it was found that the increased permeability and disrupted tight junctions of human vascular endothelium cells were effected through a mechanism partially dependent on MCP-1, which was secreted by DV2-infected monocytes and lymphocytes.

INTRODUCTION

Dengue viruses (DV), which belong to the genus Flavivirus, may cause symptomatic infection with clinical syndromes that include the mild, undifferentiated febrile illness called classical dengue fever (DF) and a more severe disease, the potentially fatal, four-level, plasma-leakage syndrome known as dengue haemorrhagic fever (DHF) (grades I and II) or dengue shock syndrome (DSS) (grades III and IV) (Halstead, 2002; Monath, 1994). The hallmarks of DHF and DSS are vascular leakage, thrombocytopenia and haemo-concentration (Bhamarapravati, 1989; Kalayanarooj et al., 1997; Nimmannitya, 1987; Rothman & Ennis, 1999).

As the endothelium forms the primary barrier of the circulatory system, dysfunction of the endothelial cells during acute diseases can broadly affect vascular permeability and cause plasma leakage. Although DV-infected human endothelial cells undergoing apoptosis have been reported (Avirutnan et al., 1998; Bunyaratvej et al., 1997; Diamond et al., 2000; Huang et al., 2000), no virus has been detected in the endothelial cells from biopsy skin samples (Andrews et al., 1978; Jessie et al., 2004; Killen & O'Sullivan, 1993; Sahaphong et al., 1980). One of the most severe symptoms of DV-related inflammation is vascular leakage, which has been linked to mediators secreted by cells in the circulatory system (Rothman & Ennis, 1999).

Monocytes and macrophages have been demonstrated to be the targets of DV infection in the circulatory system, both in vitro and in vivo (Halstead & O'Rourke, 1977; King et al., 1999; Klits, 1990; Kurane et al., 1990; O'Sullivan & Killen, 1994; Rothman & Ennis, 1999). In culture, DV replicates in monocyte-derived macrophages and monocyte-like cell lines. DV infection of the cells is not cytolitic, but alters the secretion of cytokines from infected cells (Bosch et al., 2002; Carr et al., 2003; Chang & Shaio, 1994; Hober et al., 2000).
Many permeability-related modulators in endothelial cells regulate the endothelium to function as an active barrier between blood and tissue. For example, increasing levels of circulating vasoactive factors, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8 and human cytotoxic factor (hCF) have been reported in DV-infected patients (Bosch et al., 2002; Chang & Shaio, 1994; Hobert et al., 1996). hCF is produced by DV-infected T cells and induces leakage in the peritoneal cavity of mice (Khanna et al., 1990).

In this study, we propose the chemokine monocyte chemoattractant protein-1 (MCP-1) as a novel candidate modulating vascular permeability in DHF/DSS patients. MCP-1, a member of the CC chemokine family, is critical for directing the extravasation of mononuclear cells into inflamed, infected and traumatized sites (Leonard & Yoshimura, 1990).

**METHODS**

**Cells and culture.** Human blood from healthy donors was collected and separated into peripheral blood mononuclear cells (PBMCs) and neutrophils using a density gradient kit (Nycosrep 1.077; Life Technologies). Monocytes in the PBMCs were then absorbed onto to serum-coated culture flasks for 2 h. Adherent mononuclear cells were detached from the flask and resuspended in RPMI 1640 containing 5 % autologous plasma (Gibco). Isolated cells were plated in six-well culture plates at a density of 2 × 10^6 cells per well. Cell viability was determined using trypsin blue exclusion and the purity of monocytes was confirmed using flow cytometry with anti-human CD14 antibody. Only cell preparations with 95 % viability and 90 % purity or greater were used. Human umbilical vein endothelial cells (HUVECs) were collected from umbilical cord veins using a procedure described previously (Gamble et al., 1985). Briefly, cells isolated from an individual umbilical cord were grown in Medium 199 (Life Technologies) supplemented with 10 % fetal calf serum (FCS; Life Technologies), 2 mM l-glutamine (Life Technologies), 100 U penicillin ml^-1^ and 100 μg streptomycin (Life Technologies) ml^-1^ at 37 °C in an incubator with humidified air and 5 % CO₂. Only passage-two HUVECs were used.

**Patient sera.** Dengue patient sera were kindly provided by Dr N. T. Hung (Department of Dengue Haemorrhagic Fever, Children’s Hospital No. 1, Ho Chi Minh City, Vietnam). Forty-four serum samples were collected from patients with DF and DHE (grades A1 and II) or DSS (grade III and IV). The diagnosis of DHE was based on the clinical criteria established by the World Health Organization. Twenty-seven serum samples were collected from patients with enterovirus 71 (EVT1) infection, kindly provided by Dr C.-C. Liu (Department of Pediatrics, National Cheng Kung University Medical Center, Tainan, Taiwan). Sera from healthy volunteers were used as negative controls.

**DV infection of monocytes.** Healthy human monocytes in 10 cm culture plates were trypsinsized and resuspended in RPMI 1640. Cells (2 × 10^6) were seeded into each well of a six-well culture plate. After overnight incubation, DV or heat-inactivated DV (56 °C, 30 min) was added to the monocytes at an m.o.i. of 1 and incubated at 37 °C for 2 h (Habot-Wilner et al., 2005). The culture medium was then removed and replaced with fresh growth medium (1:5 ml per well). The conditioned media were harvested at various time points post-infection (p.i.), processed through a 0.22 μm filter and stored in a freezer at −70 °C.

**RT-PCR.** Expression levels of MCP-1 and β-actin mRNA were determined using RT-PCR analysis. Total cellular RNA from 1 × 10^6 HUVECs, hepatoma Hep3B cells or monocytes was extracted using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. The concentration of RNA was determined using a spectrophotometer at a wavelength of 260 nm (U-2000; Hitachi). cDNA was prepared using total RNA (1 μg) extracted from various cells and reverse transcription, as described previously (Lin et al., 2000), using a GeneAmp PCR System 9600 (Perkin-Elmer). The PCR was conducted in 50 μl reaction mixture (1:5 mM MgCl₂ and 0.2 mM each of dATP, dGTP, dCTP and dTTP) containing primers at 1:5 μM each, 0-2 μg RNase A (Sigma-Aldrich) ml^-1^, 1 μl cDNA template and 1 U Taq DNA polymerase (Promega). The PCR program was as follows: 94 °C for 1 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; and a final cycle at 72 °C for 5 min. The oligonucleotide primers used for recombinant human MCP-1 (rhMCP-1) (sense, 5’TCCCCCGGGAGAATCCTGCCGCGGATGAAAGTCTCTGCCGCCC-3’; antisense, 5’CCGCTCCAGTCAAGTCTTGGAGTTGG-G-3’) and β-actin (sense, 5’TGGAAATCTGTGGGATCATGATGAAAC-3’; antisense, 5’TAAACCGAGCTCAGTACAGTCGCG-3’) were according to previously published sequences (Li et al., 1993; Lin et al., 2000).

**ELISA.** MCP-1 in the culture supernatants and sera (control, EV71-infected and DHF patients) was detected using ELISA kits (R&D Systems) according to the manufacturer’s instructions. The concentration of MCP-1 was measured by spectrophotometry at a wavelength of 450 nm using an ELISA reader (Molecular Devices).

**Assay of endothelial-cell permeability changes.** HUVECs were isolated from umbilical cords (12–24 h old) using 0.05% collage-nase digestion (Sigma-Aldrich). Passage-two endothelial cells were seeded onto to membranes (Transwell Clear) with 0.4 μm pores (5 × 10^4 cells per well) and cultured in endothelial basal medium-2 (EBM-2; Cambrex Bio Science Walkersville) accompanied by endothelial growth medium-2 (EGM-2; Cambrex Bio Science Walkersville). The Transwell device consists of an upper chamber formed by the membrane contained within an insert that is placed inside the well of a 24-well plate and a normal lower chamber. On day 1 post-plating, the medium in the upper chamber was changed to Opti-MEM (Gibco) containing 2% FCS (v/v). On day 2 post-plating, the assay was carried out as follows: the medium in the upper chamber of the Transwell was replaced with 150 μl fresh Opti-MEM (serum-free) plus 30 μl mock-infected or DV2-infected monocyte-culture supernatant. The cells were then incubated for 3 h at 37 °C. Next, 15 μl streptavidin–horseradish peroxidase (HRP) (HRP (R&D Systems) diluted 1:6:1 with serum-free Opti-MEM was added to the upper chamber of the Transwell and the medium was collected from the lower chamber (20 μl) at 15 min after adding streptavidin–HRP. Samples were assayed for HRP activity with o-phenylenediamine dihydrochloride (R&D Systems) as the substrate using a colorimetric assay and the concentrations were determined using an ELISA reader at a wavelength of 450 nm. Neutralizing-antibody suppression of permeability change was analysed by pre-incubating the tested media with the antibody at 4 °C for 30 min.

**Immunofluorescence staining of ZO-1 protein.** To study the effect of MCP-1 on the distribution of the endothelial tight junction protein ZO-1, untreated HUVEC monolayers were seeded on to coverslips and treated with or without rhMCP-1 (R&D Systems) or DV2-infected monocyte-culture supernatant at 37 °C for 5 h. After three washes with PBS, all monolayers were fixed at room temperature in PBS containing 2% paraformaldehyde for 10 min. After three washes with PBS, cells were permeabilized at room temperature for 5 min with 0.1 % Triton X-100 in PBS. Cells were also
RESULTS

MCP-1 is expressed at high levels in the sera of DHF patients

We collected sera from 44 DV-infected patients, 27 EV71-infected patients and 14 healthy controls to measure the expression levels of MCP-1. MCP-1 levels in the plasma of DF and DHF patients were increased significantly compared with those in EV71-infected patients and healthy controls (Fig. 1; Table 1). In addition, the expression level of MCP-1 in DHF patients (2065–2800 pg ml$^{-1}$) was higher than that in DF patients (919–1837 pg ml$^{-1}$). However, expression levels of MCP-1 in DHF grade I to grade IV patients were not significantly different (Fig. 1). Our results demonstrate that DV specifically upregulated MCP-1 expression in the sera of the three patient groups and that DHF patients (grades I–IV) showed the highest levels of MCP-1 expression.

Transcriptional upregulation of MCP-1 in DV-infected monocytes

To determine which cell types can be induced to overexpress MCP-1, we determined the levels of MCP-1 mRNA expression in healthy human monocytes, Hep3B cells and HUVECs following infection with DV2. MCP-1 mRNA expression in DV2-infected, heat-inactivated DV2-infected and mock-infected cells (medium treatment only) were determined using RT-PCR. At the same time, we measured the percentage of DV2 infection of monocytes (95–100 %), Hep3B (95–100 %) and HUVECs (70–85 %) by immunofluorescence staining (data not shown). There was a dramatic increase in MCP-1 mRNA levels in DV2-infected monocytes compared with mock-infected and heat-inactivated DV2 groups (Fig. 2a, b). No MCP-1 expression was detected in Hep3B cells or HUVECs. In contrast, expression of another chemokine, RANTES, was elevated in DV2-infected HUVECs (detected using RT-PCR; data not shown). Our data indicated that active DV2 infection can induce mRNA expression of the highly monocyte-specific MCP-1.

Detection of MCP-1 secretion by DV2-infected monocytes

Based on the above results, we next measured expression of the secreted form of MCP-1 protein in DV2-infected monocytes using ELISA. We initially determined the MCP-1 expression levels at different multiplicities of DV2 infection. MCP-1 expression was highest following DV2 infection at an m.o.i. of 1 and then remained at a plateau for higher multiplicities (Fig. 3a). The supernatants of monocytes infected with DV2 at an m.o.i. of 1 were collected at 24, 36, 48, 60 and 72 h.p.i. and analysed by ELISA. Consistent with the results in Fig. 2, a significant increase in MCP-1 protein expression was detected in the supernatants of DV2-infected monocytes from 36 to 72 h.p.i. (Fig. 3b). The highest expression level of MCP-1 in DV2-infected monocytes occurred at 36 h.p.i. The decrease in MCP-1 expression at 48 h.p.i. was caused by extensive cell death. We detected no significant differences in MCP-1 expression levels between monocytes that were mock-infected or infected with heat-inactivated DV2. Although we detected MCP-1 expression in both DV2-infected and stimulated healthy human B and T cells, these expression levels were lower than in monocytes (data not shown). Our data clearly demonstrated that DV2 infection induced monocytes to secrete MCP-1 and that MCP-1 expression reached its highest level at an m.o.i. of 1 at 36 h.p.i.

DV2-infected monocyte-culture supernatant induces permeability alterations in the HUVEC monolayer

We assayed the permeability change in HUVECs using a Transwell system as described previously (Gamble et al., 1985). To determine the optimal exposure time of HUVEC monolayers to the supernatant of DV2-infected monocytes, we exposed HUVEC monolayers for various times to culture supernatant from monocytes that had been infected with DV2 for 36 h. The highest HRP activity, indicating a change in permeability, was detected in HUVEC monolayers exposed for 3 h (Fig. 4a). HUVECs were then exposed for 3 h to the supernatants from DV2-infected monocytes collected at various times p.i. The highest level of

Fig. 1. Evaluation of MCP-1 expression levels in the sera of DV- and EV71-infected patients. MCP-1 expression was measured in the sera of 14 healthy controls (normal), four patients with DF, 40 patients with DHF (grades I–IV) and 27 patients with EV71 infection by ELISA assay.
permeability was detected at 36 h p.i. in DV2-infected monocytes (Fig. 4b). Moreover, the permeability increase was dependent on the multiplicity of DV2 infection (Fig. 4c). Heat-inactivated DV2 affected the permeability of HUVECs only slightly, confirming that DV2 infection of monocytes caused the permeability change. Thus, our data indicate that factors in the culture supernatant of DV2-infected monocytes are responsible for the permeability change in HUVECs.

Both rhMCP-1 and conditioned medium from DV2-infected monocytes increases the permeability of HUVECs

Rat endothelial MCP-1 induced by vascular endothelial growth factor (VEGF) has been shown to cause vascular leakage in vivo (Yamada et al., 2003), indicating the

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### Table 1. Clinical and laboratory measurements of DV-infected and EV71-infected patients after diagnosis at admission, by disease

<table>
<thead>
<tr>
<th>Category</th>
<th>Healthy-control group (n=14)</th>
<th>Dv-infected patients, by disease</th>
<th>EV71-infected patients, by disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF (n=4)</td>
<td>DHF (n=30)</td>
<td>DSS (n=10)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>25±9±2.8</td>
<td>10±8±1.5</td>
<td>10±4±3.2</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>6/8</td>
<td>2/2</td>
<td>16/14</td>
</tr>
<tr>
<td>Fever day on admission</td>
<td>NA</td>
<td>1.0±0.0</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>MCP-1 (pg ml⁻¹)</td>
<td>48±7±100.3</td>
<td>1252.0±436.3*</td>
<td>2294.1±204.7*</td>
</tr>
</tbody>
</table>

*P<0.01; †P>0.05; all compared with the healthy-control group.
involvement of MCP-1 in permeability. Consistent with this, we found a correlation between the degree of permeability change in a HUVEC monolayer after coculturing with DV2-infected monocyte-culture medium and the kinetics of MCP-1 expression in DV2-infected monocyte-culture medium (Figs 3b and 4b). Therefore, we wanted to clarify whether the permeability change in HUVECs was caused by MCP-1 in the culture medium of DV2-infected monocytes. The increased permeability of HUVECs correlated in a dose-dependent manner with treatment with rhMCP-1 (Fig. 5a). This effect could be reversed by pre-incubating rhMCP-1 with MCP-1-neutralizing antibody, but not with control IgG1 antibody (Fig. 5a). We also found that conditioned medium pre-treated with MCP-1-neutralizing antibody, control IgG1 or anti-VEGF antibody for 30 min. HRP activity was measured after 3 h co-culture with HUVECs. The conditioned medium was collected at 36 h after DV2 infection (m.o.i. = 1) of monocytes. Data are displayed as the mean ± SD from three independent experiments.

**Fig. 4.** Detection of the effect of DV2-infected monocyte culture supernatant on the permeability of HUVECs. (a) HUVEC monolayers were grown in Transwells and then treated for the indicated times with the culture supernatants of monocytes infected with DV2 or mock-infected for 36 h. Changes in permeability of the endothelial-cell monolayer were assessed by measuring HRP activity (see Methods). (b) HUVEC monolayers were exposed to the culture supernatant of DV2-infected monocytes (m.o.i. = 1) for 3 h. The culture supernatants used were collected from the media of infected monocytes at various time points p.i. as indicated. (c) The endothelial-cell monolayer was treated for 3 h with the culture supernatants collected from monocytes infected with DV2 at various m.o.i. for 36 h. Data are displayed as the mean ± SD from three independent experiments. Empty bars, mock; filled bars, DV2; shaded bars, iDV2.

**Fig. 5.** Determination of factors in the culture medium of DV2-infected monocytes that cause permeability changes in HUVECs. (a) HUVEC monolayers were exposed to various amounts of rhMCP-1. MCP-1-neutralizing antibody was used to block the effect of MCP-1. IgG1 was used as a negative control. Culture supernatants were collected after 3 h and HRP activity was measured. (b) DV2-infected monocyte-culture supernatants were pre-incubated with MCP-1-neutralizing antibody, control IgG1 or anti-VEGF antibody for 30 min. HRP activity was measured after 3 h co-culture with HUVECs. The conditioned medium was collected at 36 h after DV2 infection (m.o.i. = 1) of monocytes. Data are displayed as the mean ± SD from three independent experiments.
MCP-1 in the culture medium of DV2-infected monocytes causes reorganization of the HUVEC tight junction protein ZO-1

Disarray of tight junction proteins and actins has been shown to cause endothelial permeability change (Lum & Malik, 1996) and MCP-1-induced tight junction openings in brain endothelial cells via a small GTPase Rho and a Rho kinase (Stamatovic et al., 2003). Therefore, we explored whether MCP-1 from DV2-infected monocyte-culture medium could perturb the tight junction by observing the distribution of the endothelial-cell tight junction protein ZO-1. Immunofluorescence staining showed that there was no perturbation of the HUVEC tight junction following mock treatment (Fig. 6a), but that perturbation was apparent in HUVECs treated with rhMCP-1 (Fig. 6b), DV2-infected monocyte conditioned medium (Fig. 6d) and DV2-infected culture medium combined with control IgG1 antibody (Fig. 6f). In contrast, pre-treatment of DV2-infected monocyte conditioned medium with MCP-1-neutralizing antibody reversed the change in ZO-1 distribution (Fig. 6e). Direct DV2 infection of HUVECs for 3 h did not affect ZO-1 distribution (Fig. 6c). Taken together, our results show that MCP-1 in the DV2-infected monocyte-culture supernatant perturbs the distribution of the tight junction protein ZO-1 in HUVECs.

DISCUSSION

In contrast to several in vitro studies reporting that DV replicates in endothelial cells (Andrews et al., 1978; Bosch et al., 2002; Chen et al., 1996; Talavera et al., 2004) and causes structural alteration, damage and apoptosis of endothelial cells (Avirutnan et al., 1998; Sahaphong et al., 1980), we found no evidence of DV replicating in or causing damage to the endothelium in patients, although viral antigens have been detected in biopsies of lung, liver and brain vascular endothelium of patients (Bhoopat et al., 1996; Hall et al.,

![Fig. 6. Immunofluorescent analysis of perturbation of the tight junction protein ZO-1 on the outer membrane of HUVECs cultured with DV2-infected monocyte-culture supernatant. HUVECs were treated for 3 h with culture medium only (a), rhMCP-1 (b), DV2 (m.o.i. = 1) (c) or DV2-infected monocyte-culture medium (d), or with DV2-infected monocyte-culture medium pre-treated with MCP-1-neutralizing antibody (e) or with control IgG1 (f) for 30 min. The tight junction protein ZO-1 was labelled using anti-ZO-1 antibody and observed under a laser confocal scanning microscope. Arrows indicate ZO-1 distribution.](image)
Avirutnan et al. (1998) first reported MCP-1 overexpression in the sera of DSS patients. However, the role of MCP-1 in the pathogenesis of DV infection has not been determined. We found that MCP-1 overexpression occurred at a significantly higher level in DV-infected patients than in EV71-infected patients (Fig. 1 and Table 1). Furthermore, MCP-1 expression levels were much higher in DSS patients than in DF patients, EV71-infected patients or normal controls.

DV infection inducing MCP-1 overexpression was also cell-type specific and one of the major cell types of MCP-1 production was monocytes (Fig. 2). As antibody-dependent enhancement increases the infection levels of monocytes infected with different DV serotypes (Halstead & O'Rourke, 1977), we conjecture that it might subsequently increase MCP-1 expression. In addition, Lin et al. (2005) demonstrated that anti-DV-NS1 antibody bound to vascular endothelial cells and induced MCP-1 overexpression, which upregulated ICAM-1 expression and increased the adherence of PBMCs to the anti-DV-NS1 antibody-bound endothelial cells. Whether MCP-1 released from DV-infected monocytes also upregulates the adherence of molecules expressed on endothelial cells requires further exploration.

We found that the distribution of the endothelial-cell tight junction protein ZO-1 on the cell membrane was disrupted. We hypothesize that patients infected with DV combined with another disease that induces MCP-1 expression are at greater risk of progressing to DSS than DV patients without such a complication.

ACKNOWLEDGEMENTS

This work was supported by grant NHRI-CN-CL9303P from the National Health Research Institutes, Taiwan. We acknowledge the editorial assistance of Bill Franke.

REFERENCES


Y.-R. Lee and others


