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Macrophage migration inhibitory factor induced by dengue virus infection increases vascular permeability

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ABSTRACT

Dengue virus (DENV) infection can cause mild dengue fever or severe dengue hemorrhage fever (DHF) and dengue shock syndrome (DSS). Serum levels of the macrophage migration inhibitory factor (MIF) have been shown to be correlated with severity and mortality in DENV patients, but the pathogenic roles of MIF in DHF/DSS are still unclear. Increase in vascular permeability is an important hallmark of DHF/DSS. In this study, we found that DENV infection of the human hepatoma cell line (Huh 7) induced MIF production. Conditioned medium collected from DENV-infected Huh 7 cells enhanced the permeability of the human endothelial cell line (HMEC-1) which was reduced in the presence of a MIF inhibitor, ISO-1 or medium from DENV-infected MIF knockdown Huh 7 cells. To further identify whether MIF can alter vascular permeability, we cloned and expressed both human and murine recombinant MIF (rMIF) and tested their effects on vascular permeability both *in vitro* and *in vivo*. Indirect immunofluorescent staining showed that the tight junction protein ZO-1 of HMEC-1 was disarrayed in the presence of rMIF and partially recovered when cells were treated with ISO-1 or PI3K/MEK–ERK/JNK signaling pathway inhibitors such as Ly294002, U0126, and SP600215. In addition, ZO-1 disarray induced by MIF was also recovered when CD74 or CXCR2/4 expression of HMEC-1 were inhibited. Last but not least, the vascular permeabilities of the peritoneal cavity and dorsal cutaneous capillary were also increased in mice treated with rMIF. Taken together; these results suggest that MIF induced by DENV infection may contribute to the increase of vascular permeability during DHF/DSS. Therapeutic intervention of MIF by its inhibitor or neutralizing antibodies may prevent DENV-induced lethality.

1. Introduction

Dengue viruses (DENV serotypes 1–4) are mosquito-borne flaviviruses which are widespread in the western pacific, Southeast Asia and South America [1,2]. It is estimated that over 50–100 million DENV infections occur globally each year [3]. DENV infection can lead to a flu-like illness which is called dengue fever and more severe or even fatal dengue hemorrhage fever (DHF) or dengue shock syndrome (DSS). DHF is a severe febrile disease characterized by abnormalities in homeostasis and increased capillary leakage that can progress to blood pressure decrease, and hypovolemic shock (DSS) [4]. Neither vaccines nor specific antiviral drugs are available to prevent or treat DENV infection at the present time and the mortality rate of DHF/DSS can be as high as 20% if untreated. Therefore, a better understanding of the mechanism to induce vascular leakage by DENV is necessary to develop a more effective and specific therapy against the development of DHF/DSS.

Vascular permeability of capillaries depends on the ultrastructure of the endothelial cells and the junction between these cells. Increased capillary permeability can be due to damage to capillary endothelial cells or due to various vasoactive mediators. Since no apparent endothelial damage has been found in DHF/DSS patients, it is generally believed that overproduction of cytokines and soluble mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and MMP (matrix metalloproteinase) are involved in the molecular mechanisms leading to the permeability increase in DHF/DSS [5–8].

Macrophage migration inhibitory factor (MIF) was originally described as a factor produced by T cells upon antigen stimulation that inhibits the random migration of macrophages. More recently, however, we have learned that MIF is constitutively expressed

Abbreviations: DENV, dengue virus; DHF, dengue hemorrhage fever; DSS, dengue shock syndrome; MIF, macrophage migration inhibitory factor; GFP, green fluorescent protein; shRNA, short hairpin RNA; PBMC, peripheral blood mononuclear cells; p.i., post-infection.

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inside of almost all kinds of cells and released in response to a variety of stimuli [9]. Once released, MIF can stimulate monocyte to secrete other pro-inflammatory cytokines such as TNF-α and counteracts the anti-inflammatory action of glucocorticoids [10]. It has been shown that MIF is a pluripotent cytokine with enzymatic tautomerase activity which can be blocked by its inhibitor ISO-1 [11–13]. Since MIF has a broad range of immune and pro-inflammatory activities including the increase of the leukocyte–endothelial interactions via the promotion of adhesion molecules expression on monocytes and endothelial cells, it plays very important roles in many different diseases [9,12,14–16]. In our previous study, serum levels of MIF were found to be correlated with disease severity in adult dengue patients in southern Taiwan [17]. Furthermore, MIF knockout ($Mif^{-/-}$) mice show less hemoconcentration and lethality compared with normal mice during DENV infection [18]. However, the role of MIF in causing plasma leakage in DHF/DSS is unclear. It is known that MIF can induce the expression of many inflammatory molecules such as TNF-α, IL-1β, MMP, and vascular endothelial growth factor (VEGF) from different cells. All these molecules can contribute to the vascular permeability increase during DENV infection [13,19,20]. However, it is still unclear whether MIF can directly increase vascular permeability. In this study, we investigated this question using both *in vitro* and *in vivo* vascular permeability models to test whether MIF can contribute to the increase of vascular permeability in DENV infection.

2. Materials and methods

2.1. Cells

The human microvascular endothelial cell line (HMEC-1) was cultured in MCDB 131 (Sigma–Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratory, Logan, UT), 4 mM L-glutamine, 1 ng/ml hydrocortisone and 10 ng/ml epidermal cell growth factor at 37 °C in a 5% CO_2 atmosphere. Human hepatoma cells (Huh 7) were cultured in DMEM (HyClone) supplemented with 10% heat-inactivated FBS, and 2 mM L-glutamine. Human peripheral blood mononuclear cells (PBMC) were isolated from volunteers' blood by Histopaque 1077 (Sigma–Aldrich) grown in RPMI 1640 (HyClone) supplemented with 10% heat-inactivated FBS, streptomycin 100 ng/ml, and penicillin 100 U/ml. Cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

2.2. Preparation of virus stocks, titration, and infection

Virus production and titration of the DENV-2 PL046 strain were performed as described in a previous study [21]. Virus inactivation was performed as indicated previously by exposure to short-wave UV-light [21]. Huh 7 Cells (1×10^6) were infected with DENV with multiplicity of infection (moi = 1 or 10). The infectivity of DENV to Huh 7 cells was confirmed using a mouse anti-E (50–2) monoclonal antibody (1:1000 dilution) followed by an Alexa 594-conjugated goat anti-mouse IgG monoclonal antibody (Invitrogen, Camarillo, CA) (1:200 dilution). The fluorescent intensity was detected using flow cytometry. In brief, the cells were trypsinized and immunofluorescent stain was performed as described below. The cells were finally aliquot in 1 ml of FACS buffer (BD Biosciences, Mountain View, CA) and analyzed in a FACScan flow cytometer (BD). A total of 10,000 events were collected from each sample and analyzed by WinMDI 3.0 software. In addition, the supernatants (conditioned medium) of DENV-infected Huh 7 cells were harvested at various time points post-infection (p.i.), processed through a 0.22 μm filter and stored in a freezer at −80 °C before use.

2.3. MIF silencing via short hairpin RNA

Stable clones of MIF- and green fluorescent protein (GFP)-knockdown Huh 7 cells were generated by a lentivirus-based short hairpin RNA (shRNA) system (National RNAi Core Facility, Academia Sinica, Taipei, Taiwan) targeting at sequence 5′-GACAGGGTC TACATCAACTAT-3′ for MIF or targeting at sequence 5′-GCCACAACA TCGAGGACGGCA-3′ for GFP. For lentivirus production, HEK293T cells were co-transfected with pMD.G, pCMVΔR8.91, and pLKO-based shRNA plasmid. Viruses in the medium were collected 48 h after transfection. The infected-cells were selected with a medium containing puromycin (2 μg/ml). Transient knockdown of CD74 and CXCR4 were performed using shRNAs targeting at 5′-CGCGACC TTATCTCCAACAAT-3′ for CD74 and 5′-TCCTGTCCTGCTATTGCATT A-3′ for CXCR4. For transient knockdown, HMEC-1 were seeded on six well plate at 2×10^5 cells and transfected with shRNA plasmids (1.5 μg) using PolyJet DNA transfection reagent (Signagen Laboratories, Ijamsville, MD, USA).

2.4. Recombinant MIF production

Recombinant human MIF (rMIF) and murine MIF (rmMIF) were cloned and expressed in *Escherichia coli* Rosetta using T7 polymerase-based pET-43.1a(+) vector (Novagen, Madison, WI) with $(His)_6$ tag fusion protein. Recombinant MIF was purified by Ni Sepharose (GE Healthcare, Sweden). The column was washed with 20 columns of 20 mM phosphate buffer saline (PBS), 500 mM sodium chloride, 150 mM imidazole, 0.05% Triton X-114 and eluted with 20 mM sodium phosphate buffer, 500 mM sodium chloride, 500 mM imidazole. The purified protein was analyzed by SDS–PAGE and Western blotting. In some cases, rMIF was denatured by boiling at 100 °C for 10 min.

2.5. Western blotting

For Western blotting, rMIF was detected using a 1:1000 diluted rabbit anti-MIF polyclonal antibody (Santa Cruz Biotechnology, Germany) following by a 1:10,000 diluted horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin antibody (Sigma–Aldrich). Bound HRP-conjugated antibodies were detected using Enhanced Chemiluminescence Western Blotting kit (Amersham Pharmacia Biotech, UK).

2.6. Functional assays of rMIF

For pro-inflammatory assay, PBMC (2.4×10^6 cells/ml) in a tube were treated with or without MIF and MIF inhibitor, ISO-1 (50 μM; Calbiochem, La Jolla, CA) for 6 or 24 h. The supernatants were collected for cytokines assays by enzyme-linked immunosorbent assay (ELISA) as described below. For tautomerase activity assay, L-dopachrome methyl ester was prepared at 4 mM through oxidation of L-3,4-dihydroxyphenylalanine methyl ester with 8 mM sodium *m*-periodate as previously described [22]. Tautomerase activity of rMIF was determined at room temperature by adding 0.3 ml dopachrome methyl ester to a cuvette containing 10 ng of rMIF in 0.7 ml PBS (pH 7.3). The decrease of absorbance at 475 nm was recorded from 10 to 120 s.

2.7. ELISA of cytokines

Culture supernatants were collected at different intervals p.i. as indicated. The concentrations of MIF, TNF-α, and IL-1β in the supernatants were determined with DuoSet ELISA kits (R&D Systems, Minneapolis, MN) using tetramethylbenzidine (TMB) (Clinical Science Products, Mansfield, MA) as the substrate. After