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Original article

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A R T I C L E   I N F O

Article history:
Received 30 July 2022
Received in revised form 13 September 2022
Accepted 6 October 2022

Keywords:
CD66e
CD26
CEACAM5
DPP4
PBMCs
MERS-CoV
Saudi Arabia

A B S T R A C T

Background: Middle East respiratory syndrome-coronavirus (MERS-CoV) utilizes CD26 (dipeptidyl peptidase-4) and CD66e or CEACAM5 (carcinoembryonic antigen-related cell adhesion molecule 5) receptors for cell infection. Peripheral blood mononuclear cells (PBMCs) play a critical role in mounting adaptive immune response against the virus. This study was performed to assess the expression of CD26 and CD66e on PBMCs and their susceptibility to MERS-CoV infection.

Methods: Surface expression of CD26 and CD66e receptors on PBMCs from MERS-CoV patients (n = 20) and healthy controls (n = 20) was assessed by flow cytometry and the soluble forms were determined by enzyme-linked immunosorbent assay (ELISA). MERS-CoV UPe and Orf1a genes in PBMCs were detected by using Altona diagnostics reverse transcription polymerase chain reaction (RT-PCR) kit.

Results: Mean fluorescent intensity (MFI) of CD66e was significantly higher on CD4 + lymphocytes (462.4 ± 64.35 vs 325.1 ± 19.69; p < 0.05) and CD8 + lymphocytes (533.8 ± 55.32 vs 392.4 ± 37.73; p < 0.04) from patients with MERS-CoV infection compared to the normal controls. No difference in MFI for CD66e was observed on monocytes (381.8 ± 40.34 vs 266.8 ± 20.6; p = 0.3) between the patients and controls. Soluble form of CD66e among MERS-CoV patients was also higher than the normal controls (mean= 338.7 ± 58.75 vs 160.7 ± 29.49 ng/mL; p < 0.01). Surface expression of CD26 on PBMCs and its soluble form were no different between the groups. MERS-CoV was detected by RT-PCR in 16/20 (80%) patients from whole blood, among them 8 patients were tested in PBMCs, 4/8 (50%) patients were positive.

Conclusion: Increased expression levels of CD66e (CEACAM5) may contribute to increased susceptibility of PBMCs to MERS-CoV infection and disease progression.

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https://doi.org/10.1016/j.jiph.2022.10.008
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1. Introduction

The Middle East Respiratory Syndrome coronavirus (MERS-CoV) belongs to a group of Beta-coronaviruses that first emerged in the Middle East, especially Saudi Arabia in 2012 [1]. The virus causes severe lower respiratory tract infection with up to 30% mortality, particularly in patients with co-morbidities [2], and has led to numerous hospital outbreaks [3–10]. MERS-CoV is a zoonotic infection that originated from bats [11]. Later, the virus was transmitted to humans through dromedary camels, that are believed to be the intermediate hosts [12]. To date, the precise mechanism of animal to human transmission is not clear. With MERS-CoV circulating around the Arabian Peninsula and from there spread worldwide to 27 countries, there are currently no authorized vaccines or therapeutics presently available for clinical use [13].

The clinical presentation of MERS-CoV infection ranges from asymptomatic or mild disease, to critical illness resulting in acute respiratory distress syndrome (ARDS) and multiorgan failure, the mechanisms involved in disease pathogenesis is still not fully understood [14]. A recent in-vitro study has shown that the spike protein of MERS-CoV mediates infection by binding to CD26 (dipeptidyl peptidase-4 (DPP4)) as well as CD66e (carcinoembryonic antigen-related cell adhesion molecule-5 (CEACAM-5)) receptors on host cells surfaces [15–17]. Additionally, blocking the interaction between the viral spike protein and the cell surface CD26 or CD66e receptors with specific antibodies, recombinant proteins, or small interfering RNA (siRNA) could effectively block viral cell entry [18,19]. CD26 is a co-stimulatory molecule involved in T cell activation, it is expressed on many cell types including epithelial cells of many organs including lungs, kidneys, thymus, intestine, liver, and activated T lymphocytes in bone marrow [16,17,20]. On contrary, CD66e is a member of CD66 family which has been shown to be involved in cell differentiation, cell survival and apoptosis, and expressed on epithelial cells as well as on leukocytes including T cells and monocytes [17,21–23], peripheral blood mononuclear cells (PBMCs) including lymphocytes (CD4 T cells, CD8 T cells) and monocytes play a vital role in controlling and clearing pathogens by their anti-microbial properties. They act as phagocytic and cytolytic cells. In addition, upon activation they produce various cytokines such as IL-1, IL-6, IL-2, IL-12, IL-10, and IL-18, which serve to propagate the adaptive immune responses. Moreover, they function as antigen presenting cells [24–26]. The role of PBMCs in MERS-CoV pathogenesis and subsequent spread to the internal organs remain unclear. Thus, we aimed to evaluate the expression levels of soluble and surface CD26 and CD66e receptors on PBMCs from MERS-CoV infected patients and compared them to that of healthy controls and correlated it with the detection of MERS-CoV.

2. Material and methods

A cross-sectional observational study conducted between December 2018 and December 2019 to assess the expression levels of soluble and surface CD26 and CD66e receptors on PBMCs from MERS-CoV infected patients and compared them to healthy controls. Detection of MERS-CoV infection within whole blood and PBMCs was also measured. A total of 20 (17 males and 3 females) MERS-CoV infected patients were included. The mean age of patients was 50.6 ± 17 years (range 25–93 years). Diagnosis of MERS-CoV infection was confirmed by detecting MERS-CoV S and ORF7a genes using RT-PCR. The most frequent symptom among the patients was fever in 17 (85%) patients followed by cough in 13 (65%) (Table 1).

None of the patients received any antiviral, monoclonal antibodies or immunosuppressive medications. A group of 20 healthy volunteers were included in the study to act as controls. They were all males, mean age was 36 ± 11 years (range 26–68 years). All the controls were screened for hepatitis B or C viruses (HBV, HCV), human immunodeficiency virus (HIV) and human T-lymphotropic leukaemia virus (HTLV) and were all negative. This study was approved by the Institutional Review Board (IRB) Committee, research project # E15–1625, and all patients and controls signed an informed consent.

2.1. Soluble CD26 and CD66e using enzyme-linked immunosorbent assay (ELISA)

Plasma samples were assayed for soluble CD26 and CD66e using separate ELISA kits for CD26 (ab222872, Abcam, USA) and CD66e (ab99992, Abcam, USA) in accordance with the instructions of manufacturers. Briefly, 50 μL of each sample and serially diluted standards provided were loaded into a 96-well CD26 or CD66e antibody precoated plates. This was followed by incubation for 2 hours (hrs) at room temperature. The plates were then washed 5 times with the buffer and 50 μL of biotinylated anti-human monoclonal antibody was added and incubated for 1 hr. The plates were washed again 5 times with the buffer and 50 μL of streptavidin-horseradish peroxidase conjugate was dispensed in each well and incubated at room temperature for 30 minutes (min). After washing, 50 μL of substrate solution (TMB) was added and finally the reaction was stopped using 50 μL of H2SO4 after 30 min. The colorimetric signal was measured by absorbance at 450 nm using the Anthos Zenyth 200rt microplate reader (biochrom). The limit of detection for CD26 is 50 ng/mL whereas the detection limits for CD66e is 0.2 ng/mL.

2.2. PBMC isolation and cell surface staining

Whole blood was collected in 10 mL purple top vacutainer tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ, USA). PBMCs were isolated from whole blood by density gradient centrifugation. The blood was layered on top of 4 mL of Ficoll–Paque™ Plus (GE Healthcare, Piscataway, NJ, USA) in 15 mL tubes and centrifuged for 30 min at 400g with the brake set to off. PBMCs were collected, washed twice with sterile phosphate-buffered saline (PBS), resuspended in freezing medium (fetal bovine serum (GIBCO) with 10% dimethylsulfoxide (DMSO)), and stored at −80 °C until further use.

For cell surface staining, PBMCs were thawed and washed three times with PBS. Cells were counted and distributed equally (4 × 10⁷ cells / FACS tubes). Cells were then stained with CD66e-FITC.
(Abcam), CD26-PE (BD biosciences, USA), CD4-PE-Cy7 (Abcam), CD8-APC (Abcam), CD14-PerCp (Meltini biotec, Germany) antibodies and incubated in the dark for 20 min at 25 °C. The cells were then washed once and run using the BD Biosciences LSRII flow cytometry (BD Biosciences, USA). Data were analyzed using FACS diva software (BD Biosciences, USA). For CD66e and CD26 expression on monocytes, the gating was performed based on CD14 positive within monocytes gate. For CD66e and CD26 expression on lymphocytes, the gating was performed based on CD4 positive or CD8 positive within lymphocytes gate.

2.3. RNA extraction and MERS-CoV detection using RT-PCR

Total nucleic acid extractions from PBMCs performed using the Nucleic Acid Isolation Kit I and the MagNA Pur e Compact system (Roche Applied Science) at default settings. Extractions were performed on 1 × 10^6 / 200 μL of each specimen, with a final elution volume of 50 μL. This was followed by reverse transcription of a 10 μL of the extracted RNA into cDNA using random primer. The synthesized cDNA was then amplified and screened for detection MERS-CoV genes (UpE and Orf1a) using specific primers and probes of the RealStar® altona diagnostics kit (Humburg, Germany), and Rotor-Gene Q (Qiagen, Santa Clarita, CA). All patients were confirmed to have MERS-CoV infection by detecting both UpE and Orf1a genes from nasopharyngeal swab (NPS). For detection MERS-CoV from whole blood and isolated PBMCs samples, a patient was considered positive if at least one gene (UpE or/and Orf1a) was detected.

2.4. Statistical analysis

Data were collected and statistically analyzed using GraphPad Prism 5 software. Non-paired two tails t-test was used for determination of the statistical significance between the study groups. P ≤ 0.05 was considered statistically significant.

3. Results

3.1. Evaluation of soluble and surface CD26 and CD66e receptors expression levels

We hypothesized that increased expression of CD26 and/or CD66e receptors may contribute to MERS-CoV infection and associated complications frequently observed in MERS-CoV patients such as renal failure [27–30]. Thus, the surface CD26 and CD66e receptors expression levels on PBMCs including CD4 + T cells, CD8 + T cells, and CD14 + monocytes from the study groups were assayed using flow cytometry (Figs. 1 and 2). Data for expression of CD66e are presented in Fig. 1. There was a significant increase in the expression level of surface CD66e receptors on CD4 + T cells (mean florescence intensity (MFI) = 462.4 ± 64.35, P < 0.05) and CD8 + T cells (MFI = 533.8 ± 55.32, P < 0.04) but not on CD14 + monocytes (MFI = 381.8 ± 40.34, P = 0.3) from MERS-CoV patients compared to healthy controls (CD4 + T cells; MFI = 325.1 ± 19.69, CD8 +T cells; MFI= 392.4 ± 37.73, CD14 + monocytes; MFI= 266.8 ± 20.6). Fig. 2 shows data for CD26 expression. There was no significant difference between surface CD26 expression of CD4 + T cells, CD8 + T cells, and CD14 + monocytes between MERS-CoV infected patients (CD4 + T cells; MFI= 931.9 ± 132.9, CD8 + T cells; MFI = 1134 ± 122.6, CD14 + monocytes; MFI = 883.1 ± 121.1) and healthy controls (CD4 +T cells; MFI = 819.4 ± 106.2, CD8 +T cells; MFI = 902.1 ± 144.4, CD14 + monocytes; MFI= 643.5 ± 72.23).

Fig. 3 shows data for assessment of soluble forms of CD26 and CD66e. Significant elevation of soluble CD66e among MERS-CoV patients (mean= 398.7 ± 58.75 ng/mL, P < 0.01) compared with healthy controls (mean= 160.7 ± 29.49 ng/mL) was observed (Fig. 3A). The levels of soluble CD26 receptors between MERS-CoV patients (mean= 98.22 ± 12.29 ng/mL) and the healthy controls (mean= 81.56 ± 7.525 ng/mL) were however no different (Fig. 3B).

3.2. Detection of MERS-CoV in PBMCs among MERS-CoV patients

In order to determine if PBMCs are susceptible to MERS-CoV infection, we utilized RT-PCR for detection of MERS-CoV UpE and OrfA1 genes in patient whole blood and isolated PBMCs using Altona kit. Out of 20 MERS-CoV patients tested 16 patients (80%) were positive for at least one MERS-CoV gene from whole blood, and among them 8 patients were further tested from isolated PBMCs, 4
(50%) patients were positive for at least one MERS-CoV gene.

4. Discussion

This study for the first time ever demonstrated that patients with MERS-CoV infection had increased expression of CD66e receptors on CD4 and CD8 lymphocytes compared to the normal healthy controls. Elevated expression CD66e on CD4+ and CD8+ lymphocytes was associated with increased levels of plasma soluble forms of CD66e in MERS-CoV patients as well. The increased expression of CD66e could be due to direct effect of viral antigens on different cell types including PBMCs or indirectly by the induction of cytokines. Studies have shown that the expression CD66e could be increased on the epithelial cells of the lung by IFN and bacterial respiratory infections

<table>
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<tr>
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<th>PBMCs MERS-CoV</th>
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Table 2
Detection of MERS-CoV in nasopharyngeal swab (NPS), whole blood (WB) and PBMCs.
MERS-CoV infection resulting in establishing disease severity and study. Could be detected and diagnosed from patient serum surface glycoprotein CD9 to infect human cells tested for PBMCs confirming its capability of infecting PBMCs of MERS-CoV specific memory lymphocytes due to lack of previous overexpression of CD66e in permissive cells has clearly been shown to enhance MERS-CoV attachment and entry in BHK21 cells. It is possible that CD66e may facilitate MERS-CoV entry in cells in conjunction with CD26.

No difference in CD26 expression on PBMCs or the soluble forms of CD26 was observed between the patients and controls. A recent study has shown decreased levels of soluble CD26 receptors in severe MERS-CoV patients. Another study has reported that persistent infection with MERS-CoV was associated with downregulation of CD26 expression in bat cells. Following influenza vaccination as an example of resolved successful immune response high level of CD26 expression was induced on memory CD8+ lymphocytes whereas chronic infection with persistent antigen such as cytomegalovirus (CMV), Epstein–Barr virus (EBV) or human immunodeficiency virus (HIV) lead to defective T-cell memory with low expression of CD26. High expression of CD26 is considered as a characteristic feature of memory cells. The low expression of CD26 observed in the present study could possibly be due to absence of MERS-CoV specific memory lymphocytes due to lack of previous exposure to the virus.

In the current study, we detected MERS-CoV genes in patients whole blood and isolated PBMCs. Although, MERS-CoV is a respiratory virus which targets primarily epithelial cells lining the respiratory system and alveoli, it was detected in 50% of the patients tested for PBMCs confirming its capability of infecting PBMCs through, at least in part, CD66e receptors. Recent studies have suggested that MERS-CoV could also utilize other cell surface proteins such as 78-kDa glucose-regulated protein (GRP78) and the cell surface glycoprotein CD9 to infect human cells. These molecules however, were not invested in the present study. In agreement with our result, it has been reported that MERS-CoV infection could be detected and diagnosed from patient serum. In an in-vitro study, it has been shown that MERS-CoV could infect macrophages and dendritic cells. Also, it has been found in an ex vivo study that MERS-CoV is capable of infecting T cells and inducing apoptosis. Infection of PBMCs with MERS-CoV could be a similar phenomenon to many viral infections such as HBV, HCV, HIV which have been elucidated in many studies to be involved in establishing the infection and disease progression. Thus, infection of PBMCs with MERS-CoV may play a role in disease severity and complications. In the present study, infection of PBMCs with MERS-CoV could only be tested in 8 patients due to limited availability of blood samples. Moreover, the number of patients could not be increased due to the fact that the prevalence of MERS-CoV infection globally is low and this issue is among one of the limitations of the study.

In conclusion, the increased expression levels of CD66e (CEACAM5) may contribute to increased susceptibility of PBMCs to MERS-CoV infection resulting in establishing disease severity and progression. Further investigation during different phases of the disease and the associated co-morbidities may provide a better insight into the pathogenesis of MERS CoV infection, and may help to guide targeted future therapeutics.

Declarations of interest
None.

References