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Expressão de CD64 em polimorfonucleares como potencial marcador para o monitoramento da replicação do citomegalovírus humano após transplante renal

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ABSTRACT

Human cytomegalovirus (HCMV) infection is the main cause of morbidity in kidney transplant recipients. This study aims to investigate if CD64 expression on polymorphonuclear (PMN) cells is useful for the detection of HCMV infection in eleven kidney recipients during sixty days. From the total patients, nine were positive for both pp65 antigenemia and HCMV by quantitative polymerase chain reaction (qPCR), all of which had circulating neutrophils expressing CD64 3-4 weeks prior to pp65 antigenemia peak. These results suggest that quantification of PMN CD64 together with pp65 antigenemia could be useful for the early diagnosis of HCMV after transplantation.

Key words: cytomegalovirus; IgG receptors; kidney transplantat; neutrophils.

INTRODUCTION

Human cytomegalovirus (HCMV) infection/disease cause substantial morbidity in kidney-transplant patients, especially in centers without protocol-specific follow-up[1, 2]. In immunocompromised individuals, such as transplant patients, HCMV induces clinical symptoms of flu-like syndromes or severe gastrointestinal disease[3-5]. Monitoring of HCMV in kidney transplant recipients by phosphoprotein 65 (pp65) antigenemia or HCMV deoxyribonucleic acid (DNA) tests, together with typical signs and symptoms, is critical for the appropriate diagnosis and management of clinical HCMV reactivation or primary infection[1].

CD64 (FcγRI) is a high-affinity receptor for class of monomeric immunoglobulin G1 (IgG1) and immunoglobulin G3 (IgG3), constitutively expressed on monocytes and macrophages, but minimally expressed in resting neutrophils. Inflammatory cytokines, such as interferon-gamma (IFN-γ) and granulocyte-colony stimulating factor (G-CSF), are formal inducers of CD64 on neutrophils[6-9]. HCMV proteins compete with immune complexes against several Fc receptors – such as CD64, CD16 and CD32 – thereby dampening their activation and antiviral immunity[10].

Regarding the involvement of cellular and IgG-mediated immune response in the control of HCMV infection, little is known about the use of Fc receptors in the monitoring of HCMV infection in solid organ transplantation. Mainly, the amounts of CD64 on monocytes and neutrophils has been shown to be useful for the diagnosis of bacterial infection and sepsis, and it can distinguish between bacterial infection, viral infection and inflammatory diseases when performed in combination with a marker of bacterial infection[11, 12]. In addition, CD64 expression on polymorphonuclear neutrophils (PMN) was found to be increased when infectious complications occur following solid organ transplantation[13].

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The aim of this study was to investigate the neutrophil CD64 expression in the immediate postoperative period in kidney transplant recipients in order to verify the CD64 expression as a useful laboratory tool for the detection of HCMV infection.

PATIENTS AND METHODS

We conducted an observational, longitudinal and prospective study of eleven patients undergoing kidney transplantation at the Nephrology Division of Hospital Universitário Antonio Pedro, from December 2014 to August 2015. The HCMV infection status was monitored for sixty days after transplant operation (1, 7, 30, 45, 53, 60 days) using pp65 antigenemia revealed by immunofluorescence assay and the CD64 index by flow cytometric assay.

Clinical findings and other laboratory test results were obtained from patients' records. The presence of bacterial or fungal infections was excluded by blood culture. Viral DNA was measured to validate HCMV infection at viral peak load, using the Biopur extraction kit (USA) followed by quantitative polymerase chain reaction (qPCR) performed with Alert qPCR (ELItechGroup Molecular Diagnostics Torino, Italy) according to manufacturer recommendations. This study was approved by the Research Ethics Committee of the Medical School (CAAE: 18768213.1.0000.5243, 06-09-2013).

The pp65 antigenemia was carried out by indirect immunofluorescence method[14] using the CMV Brite™ Turbo kit (IQ Products, Groningen, Netherland) according to the manufacturer's instructions. After obtaining ethylenediamine tetraacetic acid (EDTA)-treated whole blood samples from patients, leukocytes were fractionated by dextran sedimentation and erythrocytes were subsequently lysed. Cytospin slides were prepared with leukocytes cells, fixed with a formaldehyde solution, and sequentially immunostained by monoclonal antibody C10/C11, specific for pp65 protein which appears early during HCMV infection (four hours). Then a secondary antibody conjugated to fluorescein isothiocyanate (FITC) immunofluorescence was added. Positive cells were counted using fluorescence microscopy (Labomed, USA). The results were expressed as the number of positive cells per 2×10^5 leukocytes. In our center, we considered the cut-off value ten positive cells/2×10^5 leukocytes to start HCMV treatment, and the peak of HCMV infection, by pp65 antigenemia, occurred on the day 60 after kidney transplantation.

The quantification of CD64 expression on polymorphonuclear cells was performed by flow cytometry according to the datasheet from a Leuko64™ kit (Trillium Diagnostic LLC, Brewer, USA), using lyse/no-wash whole blood method containing internal calibration beads. Briefly, 50 µl of anti-coagulated blood samples were stained with CD64-fluorescein-isothiocyanate (FITC, clone 22, clone 32.2) and CD163-phycoerythrin (PE, clone Mac2-158), and then incubated for 10 minutes in the dark at room temperature. Erythrocytes were lysed by Trillium Lyse solution for 15 minutes. Subsequently, 5 µl of beads labeled with StarFire Red were added and samples were measured on a FACSCalibur flow cytometer (Becton Dickinson, NY, USA). CD163 antibody was included in the kit to differentiate neutrophils from monocytes. A gate was set around the different cell populations and the mean fluorescent intensity (MFI) was defined as the geometric mean of the logarithmic fluorescence intensity emitted by the respective cell lines. The automated software (Leuko64™ Quanti-CALC, contributed by Verity Software House, Topsham, Maine and Trillium Diagnostic, LLC, USA – version number: 1.2) for flow cytometry data analysis uses an iterative clustering to find algorithms to locate lymphocytes (internal negative control; cut-off < 1.0), monocytes (internal positive control; cut-off > 3.0), and granulocytes depending on SSC Height and CD163 PE FL-2 Height. Flow cytometry was performed within 36 hours after blood sampling. A cut-off value of 1.00 for CD64 index was adopted as suggested by manufacture. Only one researcher with training and expertise read in blind the index test and the reference standard.

RESULTS

The remaining cohort patients had a mean age of 46.5 ± 9.8 years and were 75% female. Throughout the study, 66 samples were collected. Nine patients had positive pp65 antigenemia, confirmed by Alert qPCR to HCMV (ELItechGroup, Italy), and the presence of gastrointestinal symptoms in the first three months after transplantation. Two patients never developed symptoms and had negative pp65 antigenemia in the three months after transplantation; therefore they were included in the control group. The mean time of hospitalization was 27.25 ± 15.2 days after transplantation and all patients were negative to bacterial and fungal infections during the study period.

Increased CD64 expression was observed in our patients seven days after the transplant, though the peak of CD64 expression occurred on the day 45 after transplantation. **Figure** shows the PMN CD64 expression and pp65 antigenemia profiles from five to 11 patients. An asymptomatic patient, who was negative for both qPCR and pp65 antigenemia, showed low PMN CD64 index (mean of 0.62 ± 0.11) in every sample tested until the day 60 (Figure A). On the other hand, patients with HCMV infection confirmed by positive qPCR, positive pp65 antigenemia and gastrointestinal manifestations showed PMN CD64 index > 1.0 in the 30-45 days of kidney transplantation one week before the pp65 antigenemia peak (Figure B-D).

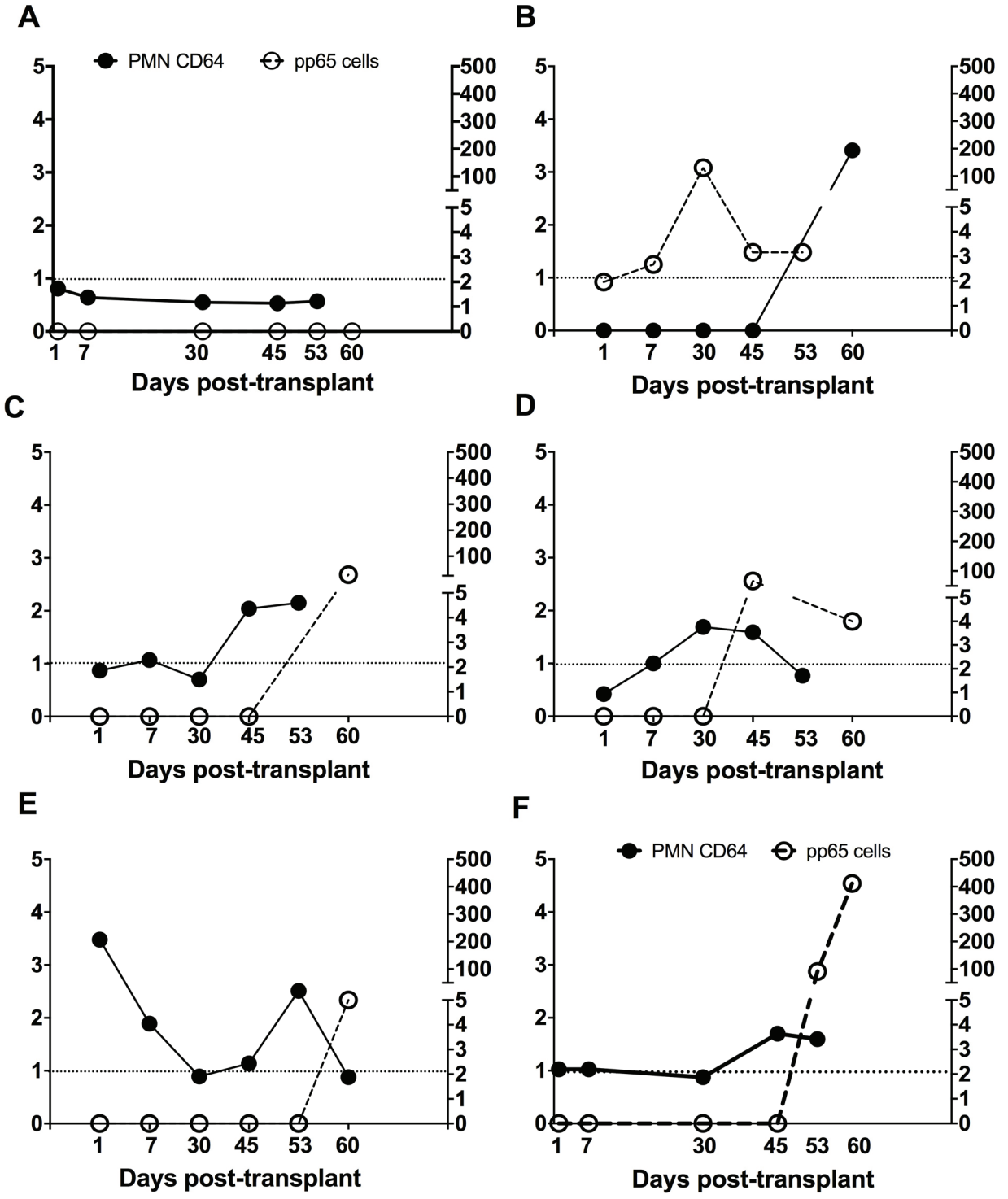

FIGURE – *Profile of PMN CD64 index and pp65 antigenemia from recipients followed for sixty days after kidney transplantation*

A) an asymptomatic recipient representative of the control group without HCMV infection; B-D) three kidney recipients that developed HCMV infection/disease after transplantation; E) kidney recipient with HCMV infection/disease, which had heart attack during surgery; F) mean value of PMN CD64 and positivity of pp65 cells of nine patients with HCMV infection/disease. The mean values of PMN CD64 expression to each point – day 1: 1.027; day 7: 1.027; day 30: 0.874; day 45: 1.700; and day 53: 1.595. Additionally, mean values of pp65 positive cells were day 1: 0; day 7: 0; day 30: 0; day 45: 0; day 53: 90.42; and day 60: 411.40.

PMN: polymorphonuclear neutrophils; HCMV: human cytomegalovirus.

Figure E shows the profile of a patient that had a heart attack during kidney transplantation. Elevated PMN CD64 index was demonstrated in the day 1 (value of 3.48) with slow decline of PMN CD64 expression until day 30. Despite of the heart attack, PMN CD64 index > 1.0 (value of 1.14) was observed 15 days before the pp65 antigenemia peak.

The Figure F exhibited the mean value of PMN CD64 expression obtained from nine patients with HCMV infection/disease. Elevated mean value of PMN CD64 expression (value of 1.7) was observed in 3-4 weeks before the appearance of pp65 peak, noticed in day 60 (mean 441.4/2 × 10^5 leukocytes) (Figure F). The PMN CD64 index assumed > 1.0 was observed in the days 1 and 7 following slight reduction in the day 30 (< 1.0). After that, there was an increase in the PMN CD64 index (value of 1.7) 45 days after transplantation. Simultaneously, the positivity of pp65 antigenemia was detected in the day 53 (approximately 100 cells) with peak in the day 60 (> 400 cells).

DISCUSSION

This pilot study was designed to investigate whether CD64 expression on PMN could predict HCMV infection after kidney transplantation. Our approach was to follow these patients for sixty days after transplantation. Our study suggests that the increased expression of CD64 on PMN cells may serve as an early detector of viral infection by HCMV. The increase in mean of CD64 expression occurred fifteen days before the appearance of HCMV replication as detected by pp65 antigenemia positivity in > 10 cells/2 × 10^5 leukocytes. Some studies have reported that the expression of CD64 on PMN surface is a good marker for diagnosis of bacterial infection in children, adults and febrile elderly patients[15-17]. However, the test does not distinguish between bacterial and viral infections, and the use of a more specific marker for each type of infection for the differential diagnosis is required[12]. In view of that, we resorted to pp65 antigenemia assay or qPCR as laboratory tools to confirm the HCMV infection[18-20].

High CD64 expression in the first seven days after transplantation is due to other comorbidities or related to the surgical procedure itself such as that exhibited by the patient that had a heart attack during the surgery (Figure E). In fact, an increase in CD64 expression in the first days after organ transplantation has already been reported, likely due to the inflammatory reaction owing to the surgical procedure[13].

Few studies have shown a relationship between the increased expression of CD64 and viral infections[12, 21, 22]. Studying 25 kidney transplant patients by Leuko64 kit, the same kit used in our study, Grey *et al*. (2011) showed that elevated PMN CD64 index was associated with infectious complications but not with graft rejection[13]. Our study is the first one to propose that increased CD64 expression in kidney transplant patients could be useful as a new tool for monitoring HCMV infection.

The use of a cut-off of 1.0 for PMN CD64 expression deserves comments. The decision was in favor of the use of 1.0 as recommended by the Leuko64 kit, but recent study suggested a cut-off of 1,115 in healthy subjects that could be used in this regard[23]. Then, more studies are necessary to establish a cut-off value.

CONCLUSION

In conclusion, our results suggest that evaluation of CD64 expression on PMN can detect the presence of HCMV infection in transplant recipients before positive pp65 antigenemia. Further studies evaluating PMN CD64 with a larger sample size are needed to confirm our results and help to validate its use as an early marker for HCMV infection after kidney transplantation, opening perspectives for effective treatment before the onset of clinical manifestations.

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AUTHOR CONTRIBUTIONS

FRC and NFR: data collection, data analysis, and writing the paper. FAP and CFS: data collection. VS: data collection, data analysis, and funding for the study. GCV: data analysis. JRL and JRA: study funding and writing the paper. AAS: funding for the study, research design, data analysis, and writing the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

RESUMO

A infecção por citomegalovírus humano (CMVH) é a principal causa de morbidade em receptores de transplante renal. Este estudo pretende investigar se a expressão de CD64 em polimorfonucleares (PMN) é útil para a detecção de infecção por CMVH em 11 receptores renais durante 60 dias. Do total de pacientes, nove foram positivos para antigenemia pp65 e para CMVH por reação em cadeia da polimerase quantitativa (qPCR), todos apresentando neutrófilos circulantes que expressam CD64 3-4 semanas antes do pico de antigenemia pp65. Esses resultados sugerem que a quantificação de PMN CD64 em conjunto com a antigenemia pp65 pode ser útil para o diagnóstico precoce de HCMV no pós-transplante.

Unitermos: *citomegalovírus; receptores de IgG; transplante de rim; neutrófilos.*

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