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Long-term genotoxic effects of immunosuppressive drugs on lymphocytes of kidney transplant recipients

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ABSTRACT

Immunosuppressive therapy can prevent rejection after organ transplantation. However, increased cancer risk is a serious complication among patients undergoing such therapy. We have evaluated whether prolonged use of immunosuppressive drugs is genotoxic. DNA instability was assessed, using the comet and micronucleus assays, in blood lymphocytes of 76 kidney transplant patients. DNA damage detected by the comet assay increased with time after transplantation. The estimated glomerular filtration rate of the patients did not influence the incidence of DNA damage. No association between micronucleated mononucleated cells and time elapsed after transplantation was observed. Our results suggest that prolonged use of immunosuppressive drugs in kidney transplant patients can induce genetic instability.

1. Introduction

End Stage Renal Disease (ESRD) patients may be treated by dialysis or undergo transplantation. Kidney transplantation is considered the best therapeutic option for those ESRD patients who are able to receive it, as it results in higher survival rates and better quality of life [1]. After kidney transplantation, patients need to take immunosuppressive medications indefinitely, to prevent immune rejection of the graft. Usually, these drugs have high pharmacokinetic ranges but narrow therapeutic indices [2]. In general, two or three immunosuppressive medications are used in combination, to minimize the risk of organ rejection [3].

Long-term use of immunosuppressive drugs is associated with severe adverse effects, such as nephrotoxicity, neurotoxicity, gastrointestinal disturbances, increased cholesterol and triglyceride levels, insulin resistance, and diabetes mellitus [4]. Among adverse effects, the development of cancer is a major cause of morbidity and mortality [5,6]. Apel et al. [7], examining the incidence of cancer in a group of 1882 German kidney transplant recipients with post-transplant times ranging 0.4–25.5 y, observed an overall incidence of 13.7%, and a risk of malignant non-skin tumors 12.1 times higher than that expected in the general population.

The long-term care of kidney transplant patients, therefore, involves a delicate balance: avoiding graft rejection, without causing excessive immunosuppression or increasing the incidence of nephrotoxicity by calcineurin inhibitors. The estimated glomerular filtration rate (eGFR), from serum creatinine, provides an adequate tool to evaluate the function of the graft, and is used routinely in clinical transplantation [8]. Monitoring the plasma concentrations of some immunosuppressive drugs is also commonly performed, in an attempt to minimize their adverse effects by adjusting the doses. However, there are no routine tests to evaluate DNA damage in transplant patients.

Some studies have assessed the mutagenic effects of immunosuppressive drugs in lymphocytes *in vitro*, using the sister-chromatid exchange [9,10], micronucleus (MN), nuclear division index (NDI) [10–12], and comet assays [12]. In general, these studies have demonstrated that high concentrations of immunosuppressive drugs can induce DNA damage. Moreover, *in vivo* studies in patients after short post-transplant periods revealed that

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the lymphocytes showed an increased incidence of sister chromatid exchanges in patients that received cyclosporine [13] and an increase in MN frequencies and a reduction in NDI after transplantation [14].

The advantage of using assays that allow monitoring of DNA stability is the prediction of the risk of cancer development and other adverse health effects induced by exposure to these immunosuppressants. Among the assays widely used for human biomonitoring, the comet assay has high sensitivity, requires small numbers of cells, and is inexpensive and quick [15]. The test can be performed concurrently with the MN assay, which evaluates mutagenic damage resulting from loss of acentric fragments or whole chromosomes [16].

According to Speit [17], the 24-h time-point after the start of the culture is ideal for analyzing MN, produced *in vivo* during division of lymphocytes in the bone marrow, thymus and lymph nodes. Therefore, mononucleated lymphocytes present *in vivo* prior to starting the culture can be analyzed for the presence of DNA damage [18], complementing the data obtained by evaluation of binucleated cells.

The aim of this study was to evaluate whether kidney transplant recipients exhibit DNA damage caused by immunosuppressive treatment and whether the degree of damage is associated with time following transplantation.

2. Materials and methods

2.1. Patients

The experiments were approved by the Ethics Committee on Human Research of the State University of Londrina, (CEP/UEL 153/2013 CAAE: 18263413.4.0000.5231). Seventy-six kidney transplant patients who regularly underwent medical monitoring at the Kidney Institute of Londrina (Paraná State, Brazil) participated in this study. Written informed consent and a questionnaire about their lifestyle were obtained from all study participants. Participants who stated that they consume alcoholic beverages were considered “drinkers” and those who stated that they smoke were classified as “smokers”. Additional data, such as post-transplantation time, rejection episodes, degree of histocompatibility with the donor, and occurrence of cancer were obtained from medical records. Venous blood samples (2 mL) were collected using EDTA (6%)-coated vacuum tubes (Labor Import, Brazil) and the coded blood samples were brought to the laboratory (stored on ice) within 2 h and processed immediately, under identical conditions. Baseline DNA damage was also estimated by MN and comet assays in 17 healthy individuals (control-group) and whole blood of three individuals was treated with methyl methanesulfonate solution (Sigma-Aldrich, CAS: 6627-3, St. Louis, MO, USA) at a final concentration of 5×10^{-5} M and incubation for 1 h at 37 °C and used as positive control in the comet assay.

2.2. eGFR and plasma concentration of tacrolimus

The patients’ eGFR values were calculated from the abridged Modification of Diet in Renal Disease formula (MDRD-4), modified by Levey et al. [19]. The transplant patients were clustered according to eGFR levels; the cutoff value used was 60 mL min^{-1} per 1.73 m^2, because GFR <60 mL min^{-1} per 1.73 m^2 for at least 3 months is evidence of kidney damage or chronic kidney disease [8,20]. Patients with eGFR >60 mL min^{-1} per 1.73 m^2 were defined as “better graft function” (BGF) and patients who showed eGFR <60 mL min^{-1} per 1.73 m^2 were defined as “worse graft function” (WGF).

For all patients studied, determination of plasma creatinine levels was obtained from the patient’s record on the same day of blood collection for mutagenicity assays. Concurrently, assessment of plasma tacrolimus levels by radioimmunoassay was also obtained from the 44 patients who were using this drug as part of their immunosuppressive regimen.

2.3. Comet assay

The alkaline comet assay was performed as described by Singh et al. [21]. Peripheral blood (20 μL) was mixed with low-melting-point agarose (Gibco, CAS: 9012-36-6, Grand Island, NY; 0.6% in phosphate-buffered saline – PBS), 120 μL. This mixture was applied to microscope slides pre-coated with normal-melting-point agarose (Life Technologies, Paisley, UK) (1.5% in PBS). Two slides were made for each patient and respective controls. The slides were immediately covered with coverslips and the agarose was allowed to solidify for 30 min at 4 °C. After removal of the coverslips, these slides were immersed in ice-cold alkaline lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 10% DMSO, 1% Triton-X) pH 10, for 24 h at 4 °C. After lysis, the slides were incubated for 20 min in alkaline buffer (200 mM EDTA, 10 N NaOH) pH 13 at 4 °C, followed by electrophoresis (25 V; 300 mA; 1 V/cm) for 20 min in the same buffer. Then, the slides were neutralized with Tris (0.4 M, pH 7.5) for 15 min, dried, and fixed with absolute alcohol for another 15 min and stored at 4 °C until analysis.

Staining was performed with GelRed Nucleic Acid Stain 10000X (Biotium, Hayward, CA), 45 μL, diluted in 0.1 M NaCl solution to 30X. A total of 300 nucleoids per patient were scored using a fluorescence microscope (Nikon Eclipse, Tokyo, Japan) with excitation filter 515–560 nm and 590 nm emission (barrier) filter, under a magnification of 400×.

The criteria used for quantification of DNA damage [22] was visual scoring of the size of the comet tail in comparison to the nucleoid. The cells were classified into four categories of DNA damage, ranging from no visible migration (class 0, undamaged cells) to the maximum length comet cells (class 3). The comet score was calculated according to the formula proposed by Manoharan and Banerjee [23], multiplying the number of nucleoids observed in each class (n0, n1, n2 and n3) by the value of the corresponding class (0, 1, 2, 3) divided by the total number of cells analyzed (N).

2.4. Micronucleus assay in mononucleated cells

The MN assay in mononucleated cells was performed according to Speit [17] by using whole blood, 600 μL, in 6 mL RPMI 1640 medium (Gibco, CAS: 31800-014, Grand Island, NY, USA), 2.0 g L^{-1} $NaHCO_3$ (Merck SA Industrias Químicas, Rio de Janeiro, RJ, Brazil), HEPES 25 mM (Sigma-Aldrich, CAS: 7365-45-9, St. Louis, MO, USA), 0.06 g L^{-1} penicillin G (Sigma-Aldrich, CAS: 113-98-4), 0.10 g L^{-1} streptomycin sulfate (Sigma-Aldrich, CAS: 3810-74-0), supplemented with 20% fetal bovine serum (Gibco, CAS: 12657-029) and 2% phytohemaglutinin A (Gibco, CAS: 10576-015) to stimulate the division of lymphocytes. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. Cells were collected by centrifugation and processed further by hypotonic treatment for 15 min in 0.075 M KCl at 4 °C. Cells were fixed three times in methanol/acetic acid (3:1). After fixation, the slides were prepared and stained with 5% Giemsa (CAS: 1.09204.0500, Merck SA Industrias Químicas) solution in phosphate buffer (0.06 M $NaHPO_4$ and 0.06 M KH_2PO_4, pH 6.8) for 10 min, washed with water and dried. Analysis under a light microscope (Nikon Eclipse E200, Tokyo, Japan) with 400× magnification was carried out to determine the frequency of micronucleated cells per 1000 mononucleated cells analyzed per patient.