Dialysis-Related Genotoxicity: Sister Chromatid Exchanges and DNA Lesions in T and B Lymphocytes of Uremic Patients

Genomic Damage in Patients on Hemodiafiltration

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Introduction
End-stage renal failure is associated with a high incidence of cardiovascular diseases and cancer and is characterized by a condition of immunodeficiency [1–9]. Among the several pathogenic mechanisms suggested to explain these phenomena there are uremia per se, micro-inflammation and oxidative stress [10, 11], which involves the whole cell structure (proteins, membrane lipids, carbohydrates and DNA) [12]. Great interest has recently been paid to oxidative DNA damage because of its genetic consequences, linked to early aging, neurodegenerative diseases, diabetes mellitus, atherosclerosis, mutagenesis and carcinogenesis [13–16].

The genome damage to the lymphocytes of peripheral blood has been widely used as a biomarker of carcinogenesis from genotoxic environmental factors, and long-term studies have demonstrated its validity and high clinical predictivity [17].

In patients with chronic renal failure the presence of massive genome damage has repeatedly been demonstrated using different methodologies, including the frequency of micronuclei [18], the single-cell gel electrophoresis [19] in PBLs, the measurement of 8-hydroxy-2-letes [20].

Abstract

Background/Aims: Patients with chronic renal failure show the presence of massive oxidative genome damage but the role played by dialysis is still a controversial issue. The aim of our study was to verify the genomic damage in B- and T-lymphocyte subpopulations of uremic patients after a single hemodiafiltration session. Methods: We enrolled 30 patients on maintenance acetate-free biofiltration and 25 age-matched healthy volunteers and studied chromosomal alterations. Results: Our data show that the basal levels of DNA damage, the number of sister chromatid exchanges and basal high-frequency cells levels are significantly higher in patients on hemodiafiltration than in controls and in T lymphocytes than in B cells. Conclusions: These findings suggest that hemodialytic treatment could represent a potential source of damage, maybe through the oxidative action of the extracorporeal circuit components, which might explain the well-known T-specific immunodeficiency correlated with uremia.
deoxyguanosine, content in the DNA of leukocytes [20], the evaluation of the sister chromatid exchange rate [16, 21]. A clear relationship between renal failure and genomic damage has been scientifically demonstrated in patients with end-stage renal disease (ESRD) on conservative therapy [19]. The role played by dialysis in this kind of genome damage is a controversial issue. A recent study reported, in patients given daily hemodialysis, levels of genomic damage lower than in patients treated with the traditional regimen of intermittent hemodialysis. Elsewhere, however, we demonstrated an increase in the genomic damage following a single session of hemodiafiltration, using highly biocompatible filters. We studied patients on maintenance acetate-free hemodialysis (Integra Physio Hospal, Bologna, Italy) with polycrylonitrile filters (AN 69-ST, Bologna Hospital, Italy) for 3.5 h, 3 times/week, and 25 age-matched healthy volunteers (13 women, 12 men; mean age 55 ± 9 years). The patients were on dialysis for primary interstitial nephritis (n = 3), nephrovascular disease (n = 5), diabetic nephropathy (n = 5), polycystic kidney disease (n = 2), and glomerulonephritis (n = 10). All the patients on dialysis were given intravenous erythropoietin 3 times/week (mean dosage 23 ± 7 IU/kg b.w.).

Kt/V, estimated following the Daugirdas equation, was maintained between 1.2 and 1.3, and the normalized protein catabolic rate equation ranged from 1.0 to 1.3 g/kg/day. All patients and control subjects were non-smokers. Among the 30 patients on acetate-free biofiltration, only 3 had tumors: 2 parathyroid adenomas (diagnosis made respectively 6 and 8 months after the uremia onset), and 1 patient had papillary carcinoma of the thyroid gland (diagnosed 3 months after the uremia onset).

The controls were not affected by any of the following conditions: cardiovascular diseases, kidney diseases, diabetes and infections. The main features of the study population are summarized in Table 1. Informed consent was obtained from all subjects. Blood samples were drawn from the cubital vein of each subject. Samples were taken from the hemodialysis patients immediately before and after the acetate-free biofiltration session and on the interdialytic day (24 h after the end of the acetate-free biofiltration session).

### Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC, obtained from 10 ml of whole blood on Histopaque 1.077 gradients, were washed twice in RPMI 1640 medium (Sigma, Milan, Italy).

### Isolation of T and B Cells

Following the manufacturer’s recommendations, the MiniMACS separation system was used to isolate T and B cells from PBMC. Briefly, PBMC were first magnetically labeled with MACS CD19 microbeads (Miltenyi Biotec) for the isolation of B cells. The cells were then passed through an MS column placed in a strong permanent MiniMACS magnet. The magnetically labeled cells were retained in the column and separated from the unlabeled cells. After removing the column from the magnetic field, the retained fraction was eluted. Both magnetic and non-magnetic fractions were recovered. The non-magnetic fraction was then labeled with MACS CD3 microbeads (Miltenyi Biotec) for the isolation of T cells from the same donor. The purity of the CD3 and CD19 cell population was determined by flow cytometry immediately after isolation.

### Limulus Test

Culture media and reagents tested for the presence of endotoxin by means of the E-toxate kit (Sigma, Italy) were found to contain ≤10 pg endotoxin/ml.

### Cell Cultures

Each cell population was then suspended in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum (Gibco), 1% non-essential amino acid solution (100×), 2 mM glutamine, penicillin/streptomycin and 1% phytohemagglutinin (Gibco). Cells were cultured at 37°C in a 5% CO2 humidified atm-
Sister Chromatid Exchanges Analysis

To prevent photolysis, the cultures were incubated for 72 h at 37°C in the dark; 24 h after starting cultures, 5-bromo-2-deoxyuridine (BrdU 0.5 µg/ml final concentration) was added. The cultures were then kept for 48 h at 37°C in total darkness. Simvastatin (Merck & Co, West Point, Pa., USA) (at concentrations of 0.1, 1 and 10 µM) or a vehicle were added to the culture vials, as described elsewhere [23]. Simvastatin was dissolved in potassium phosphate buffer, pH 7.0, before use.

We also investigated the ability of 1 mM mevalonate to reverse the effects of statin treatment, this agent being added to cultures 24 h after simvastatin.

Two hours before harvesting, Colcemid (Gibco) was added to cultures in order to arrest the cells in metaphase. To harvest cultures, cells collected by centrifugation were exposed for 20 min at 37°C to 0.075 M KCl, and fixed in freshly prepared 3:1 methanol:acetic acid. After several fixation changes, three drops of cell suspension were dropped onto microscope slides and air-dried. Slides were then stained with the fluorescence plus Giemsa method using Hoechst 33258 dye for sister chromatid exchange analysis [24].

The percentage of high-frequency cells (HFC) was estimated for each case using the pooled distribution of all sister chromatid exchange cell measurements. HFC display a number of sister chromatid exchanges per cell greater than the 95th percentile of the distribution of SCE per cell in the population [25]. For each subject, the expected number of HFC (x) was calculated in a binomial distribution to obtain the 95th percentile by means of the following formula:

\[ x = 1.645 \left[ n p(1-p) + n(1-p) + 0.5 \right]^{1/2} \]

where \( n \) is the cell number and \( p \) is equal to 0.95.

Slides from each culture were randomly numbered and scored ‘blind’ in numerical order. The mitotic index values were calculated as the percentage of cells in mitosis on the microscopic slides used for cytogenetic analysis.

Experimental data were analyzed using one-way analysis of variance (ANOVA) to determine whether there were any significant differences between values in treated subjects and in controls. Any significant differences found between treated samples and/or control samples were confirmed using Student’s t test.

Alkaline Comet Assay

Immediately after treatment, blood aliquots were collected to be processed by the Comet assay. The assay was performed essentially according to Singh et al. [26] with minor modifications, and the entire procedure was performed cooling and protecting the samples from light to avoid further DNA damage. Briefly, the blood cells were suspended in 0.5% (w/v) low melting point agarose (Bio-Rad Laboratories, Hercules, Calif., USA) and incorporated between a lower layer of 0.75% (w/v) normal melting point agarose (Bio-Rad) and an upper layer of 0.5% (w/v) low melting point agarose on microscope slides (Carlo Erba, Milan, Italy). The slides were then immersed in a lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10) containing 10% DMSO (Carlo Erba) and 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo., USA), overnight. At lysis completion, the slides were placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA, pH 13) and left in the solution for 30 min to allow DNA unwinding and alkali-labile sites expression. Electrophoresis was carried out in unwinding buffer for 25 min, 30 V (1 V/cm) and 300 mA. After electrophoresis, the slides were gently washed in 0.4 M Tris-HCl (pH 7.5).

Immediately before scoring, slides were stained with 5 µg/ml propidium iodide (Sigma Chemical Co.) and examined at 100X magnification with a Nikon E800 fluorescence microscope. 100 cells were randomly selected in each slide and scored from 0 to 4 on the basis of Comet tail length; undamaged cells (score 0) looked like an intact nucleus without tail, while damaged cells appeared as comets. Final score was calculated by the formula:

\[ \text{Comet score} = (n \text{ cells scored 1}) + (2 \times n \text{ cells scored 2}) + (3 \times n \text{ cells scored 3}) + (4 \times n \text{ cells scored 4}) \]

T and B lymphocytes from each patient were processed in triplicate. The Dunn’s test, a non-parametric multiple comparison test based on Kruskal-Wallis rank sums, was performed to analyze Comet scores.

Results

Comet Assay

The basal levels of DNA damage in uremic patients on hemodialysis were significantly higher than in the control population. The damage appeared significantly more evident in T cells than in B cells. These differences appeared even greater at the end of the hemodiafiltration session, with intermediate values at 24 h from the end of the session (fig. 1a, b).

High-Frequency Cells

Basal HFC levels in uremic patients on hemodialysis were significantly higher than those in the control population. Moreover, the dialyzed patients showed higher percentages of HFC in T-cell cultures than in B-cell cultures. These differences appeared stronger at the end of the hemodiafiltration session and even more 24 h later (fig. 2a, b).

Sister Chromatid Exchange

The number of exchanges was higher in hemodialyzed patients than in the control population. This trend increased at the end of the dialysis session, being even greater after 24 h. In all the uremic patients, the mean number of exchanges in the T-cell culture was higher than in the B-cells one (fig. 3a, b).

Dialysis-Related Genotoxicity

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**Discussion**

The present study confirms the presence of an important genome damage in uremic patients on hemodialysis, despite the use of material with high biocompatibility. This finding demonstrates that hemodialytic treatment, despite exerting a protective action against genome damage through the clearance of uremic toxins, represents a potential source of damage, maybe through the oxidative action of the extracorporeal circuit components. As already stated in the literature, ESRD patients are particularly exposed to oxidative damage [10–13, 20].

The dynamics of genome damage occurring during dialysis appear less clear-cut, the two methods used in the present analysis yielding contradictory findings. The Comet assay values showed a peak at the end of the hemodialysis session, returning to basal levels 24 h later. On the contrary, the HFC percentage and the mean SCE level, showing a significant increase at the end of the hemodialysis session, reached their highest levels 24 h after the end of the session.

The different profiles of these parameters show a later appearance of SCE maybe because of their slow production, which occurs as a late stage in a long
chain of events induced by exposure to mutagenic agents.

Conversely, Comet assay, performed at pH >13, is capable of detecting DNA single-strand breaks, alkali-labile sites and DNA cross-linking. Consequently, different stages of damage are detected by these tests: SCE, which identifies fixed mutations persisting for at least one mitotic cycle, represents a measure of DNA repair response to genome damage, while Comet is indicative of initial genome damage, in particular if repair is not allowed to occur. The usefulness of combining the two tests is to investigate both direct DNA fragmentation and chromosome/genome mutations induced by HD.

A further aspect to analyze is the different incidence of genome damage showed by B and T cells separated from the same blood sample. A real comparison between these two cellular populations, as far as dialysis-related alterations are concerned, has never been considered. T lymphocytes appear more sensitive to genome damage than B lymphocytes. Importantly, B lymphocytes seem to be less compromised by the hemodialytic stress, showing a lower level of DNA damage through the Comet assay, a lower percentage of HFC and a lower mean number of exchanges at the SCE assay with respect to T cells. These results might suggest that the humoral immune response could be less affected by a functional impairment than the cellular immune response during hemodialytic treatment. Indeed, a greater sensitivity to genome damage might explain the T-specific immunodeficiency correlated with uremia which is well documented in the literature [27]. Moreover, a minor RE% shown by the Comet assay in T cells confirmed the hypothesis of their slower repair kinetics of DNA damage.

It is now widely accepted that in patients with ESRD, immunodeficiency and an increased predisposition to cancer development coexist. Both these conditions can be related to the mutagenic action of physical, chemical and biological agents which ESRD patients are exposed to because of the inadequate clearance of endogenous substances and the contact with materials included in the dialysis circuit [27–30].

References


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