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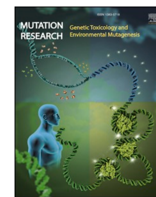

Radiosensitivity of radiotherapy patients: The effect of individual DNA repair capacity

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ABSTRACT

Individual radiosensitivity is a critical problem in radiotherapy because of the treatment restrictions it imposes. We have tested whether induction/repair of genomic lesions correlates with the acute cutaneous effects of radiotherapy. Peripheral blood samples of 56 healthy volunteers and 18 patients with breast cancer were studied. DNA damage and DNA repair capacity were assessed *in vitro* (alkaline comet assay). Patients without skin reaction did not show significant differences from healthy individuals, with respect to either initial or radiation-induced DNA damage. Similar DNA repair kinetics, fitting a decreasing exponential response, were observed in both groups, and there were no significant differences in residual genotoxic damage. In contrast, patients exhibiting acute side effects showed significantly lower DNA repair ability and significantly more residual damage, compared to patients without radiotoxicity. This approach may help to identify patients who are at greater risk of radiotherapy side effects. However, many other factors, such as dosimetry, irradiated volume, and lifestyle should also be considered in the evaluation of individual radiosensitivity.

1. Introduction

Although radiotherapy contributes to treatment of more than half of cancer patients, many individuals develop adverse normal tissue reactions, ranging from cosmetic problems to life-threatening organ dysfunctions [1,2]. While severe adverse events occur in <10 % of patients, mild to moderate effects are much more frequent [3–5]. Individual variation in radiosensitivity (RS) requires special attention. The development of clinical predictive tests has been an objective of radiobiology research for decades [6,7]. In radiation protection applications, too, it is important to understand individual variability in response, its mechanisms, and its health risk implications [3,7–9]. About 80 % of individual variability in normal tissue response is not attributable to external causes. RS is probably a complex trait depending on the interaction of multiple genes and factors [2,10,11] including stochastic variations at the molecular and cellular levels [6].

Various approaches have been used to elucidate the mechanisms of RS, from studies of the relationship between *in vitro* measurements (e.g., DNA damage, apoptosis, clonogenic survival) and *in vivo* radiotoxicity, to analysis of genetic polymorphisms [12–14]. However, in light of the complex molecular pathogenesis of the response, lack of standardization, and slowness of clinical trials, satisfactory results have not yet been achieved [13,14] and it is not clear that a single test to identify people at high risk of radiotoxicity can be developed [6].

Analysis of molecular mechanisms (e.g., DNA repair pathways) could establish their roles in this multifactorial phenotype. Reduced DNA repair capacity may be an important factor and the comet assay has been applied to test this possibility [9]. The comet assay can address important questions in radiobiology (such as tumor cell RS [12] and the bystander effect [15]), oncology, genetic toxicology [13,16,17], and human biomonitoring [18,19]. The comet assay was used to show that kidney patients with high levels of genomic damage have lower capacity to repair oxidative DNA damage [20].

Here, we have applied the comet assay to human peripheral blood samples, to examine a possible role of DNA repair capacity in adverse reactions to radiotherapy [21]. This parameter, complementing studies

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of other molecular pathways, might provide integrated information about a multifactorial trait.

2. Materials and methods

2.1. Chemicals

UltraPure™ low-melting-point (LMP) and normal-melting-point (NMP) agarose were obtained from Thermo Fisher Invitrogen (Barcelona, Spain). NaCl, Na-EDTA, Tris, NaOH, Triton X-100, Ethanol, EDTA, and DMSO were obtained from Invitrogen (CA, USA), Biopack (Buenos Aires, Argentina), and Carlo Erba Reagents (Barcelona, Spain). SYBR® Green was obtained from Sigma-Aldrich (MO, USA).

2.2. Study design and sample collection

Standardization of the comet assay is particularly important for its implementation. For this reason, we decided to develop a DNA repair curve for individuals not exposed to treatment (healthy volunteers) and then evaluate gradual adjustments for cancer patients, aiming to minimize the effects of confounding factors (e.g., age, air pollution exposure, diet, exercise, gender, infection, smoking, and alcohol consumption) [22] and mutator phenotypes (mutations in repair pathway genes, such as BRCA1/2).

The study included two groups: young, healthy female volunteers (n = 56; age, 20–35 y; mean age, 29.5 y) and sporadic breast cancer patients (n = 18; age, 42–84 y; mean age, 56.1 y). According to the recommendations [22,23], healthy volunteers were non-smokers and apparently healthy (without evidence of ongoing disease or recent infections). Thus, factors that might increase RS (smoking, diabetes, lifestyle) were excluded. To our knowledge, none of the healthy controls was previously exposed to above-background ionizing radiation.

The study was approved by the *ad hoc* Bioethics Committee of the Association of Human Genetics of Mar del Plata, Buenos Aires, Argentina. Healthy individuals and breast cancer (BC) patients gave written informed consent and answered a questionnaire on their medical history and lifestyle, including genetic diseases, medication, alcohol consumption and smoking, hypertension and diabetes, among others. Patients receiving chemotherapy prior to RT were excluded.

2.3. Blood samples

Peripheral blood samples (10 mL) of volunteers were collected in heparinized tubes by venipuncture. All samples were coded and processed within 2 h after collection. Blood samples from healthy individuals were provided by the Hemotherapy Institute of the province of Buenos Aires (Blood Bank of La Plata city), over 24 months. They were previously checked to ensure that they were free of potential biohazards (brucellosis, chagas, hepatitis B, and human immunodeficiency virus). Cancer patients were recruited at the Integrated Centre of Oncology, La Plata City, over six months. They had positive diagnosis of sporadic BC and therapeutic indication of RT after surgery to preserve the breast. Blood samples were collected before RT.

2.4. Experimental design

Samples were processed to determine DNA repair kinetics, as follows. Blood was placed into two Eppendorf tubes (1.5 mL; Hamburg, Germany). One tube was used as a control of baseline damage (without irradiation) and the other was irradiated (6 Gy) and then kept refrigerated, to prevent repair, in order to assess initial damage (t = 0). The tube was maintained at 37 °C until post-irradiation times (60 and 120 min) were analyzed. Afterwards, DNA damage was determined in all samples and at each experimental point, using the alkaline comet assay. The parameters evaluated were percentage of cells with damage, categorization of damage, and damage index.

2.5. Irradiation procedure

Cell irradiation was performed with a 4 MeV nominal accelerating potential Varian Clinac® electron linear accelerator (Varian Medical Systems, USA). 1.5 mL tubes containing sample (1 mL) were placed in an immobilized rack inside an acrylic phantom with water, whose density was equivalent to soft tissue and whose depth was greater than the buildup zone. This system was irradiated from the bottom to receive an absorbed 6-Gy dose (1.58 Gy/min). The deviation of the absorbed dose was less than 5%, which was compatible with the therapeutic objective. Although exposure in conventional RT is usually given as 2-Gy fractions, once per day, five days per week, previous results obtained in our laboratory showed that 6 Gy is optimal in relation to the sensitivity of the technique used and the detection of DNA repair [12,24].

Radiotherapy treatment was also performed with a VarianClinac® linear accelerator. All sporadic BC patients received an identical radiotherapy protocol: tangential irradiation of the whole breast, with lateral and medial wedge fields. The regimen comprised total dose 50-Gy, fractionated: 2 Gy, five times per week. The early cutaneous reaction to RT that develops within the irradiation field was evaluated by the same specialist radiation oncologist and used as an indicator of clinical RS according to the Radiation Therapy Oncology Group (RTOG) score. Thus, six degrees were considered for the radiotoxicity reactions: (0) none, (1) mild, (2) moderate, (3) severe, (4) life-threatening, and (5) death [7,8].

2.6. Comet assay

We used the alkaline comet assay according to Singh et al. [25], with minor modifications [26]. Electrophoresis was performed at 4 °C, 25 V (1.6 V/cm), 250 mA for 20 min.

2.7. Microscopic analysis and scoring

Slides were scored under a blind code. They were stained with SYBR®Green (1/2000 dilution, 20 μL) just before analysis. 100 randomly captured cells per slide were visually analyzed under a fluorescence microscope (Olympus BX40®, Tokyo, Japan) at 400 × magnification. Each experimental point was evaluated in duplicate. The degree of damage was determined visually according to the comet tail extension [27,28]. Each cell was classified into five classes, from class 0 (no DNA migration) to class 4 (maximum DNA migration). Damage level was categorized as null (comet grade 0), low or moderate (comet grades 1 and 2), or severe (comet grades 3 and 4 and apoptotic cells). Genetic damage was measured with the Damage Index (DI) in arbitrary units (AU) as $DI = \sum PDG \times DG$, where DG is the degree of damage (from 0 to 4) and PDG is the percentage of cells showing each type of damage; this index varies from 0−400 AU [14].

2.8. Statistical analysis

Two separate electrophoretic runs were carried out for each sample and the data obtained were analyzed together. 200 cells were observed per experimental point. Results from both study groups (healthy individuals and BC patients) were averaged and used for mathematical adjustment. The statistical analysis was performed with Shapiro-Wilk test and Mann Whitney Wilcoxon test a posteriori, and the chi-square test was used to analyze damage class.

3. Results

Radiation (6 Gy) significantly increased DI levels in cells from the healthy group from 11.3 ± 18.7 (SEM: 2.5) to 203.3 ± 77.1 (SEM: 10.4); ($P < 0.001$; Fig. 1A). After irradiation (60 and 120 min), DI was significantly reduced compared to t = 0 (72 and 85 %, respectively; $P <$ 0.001). This trend was seen in 96 % of individuals. Similar behaviour

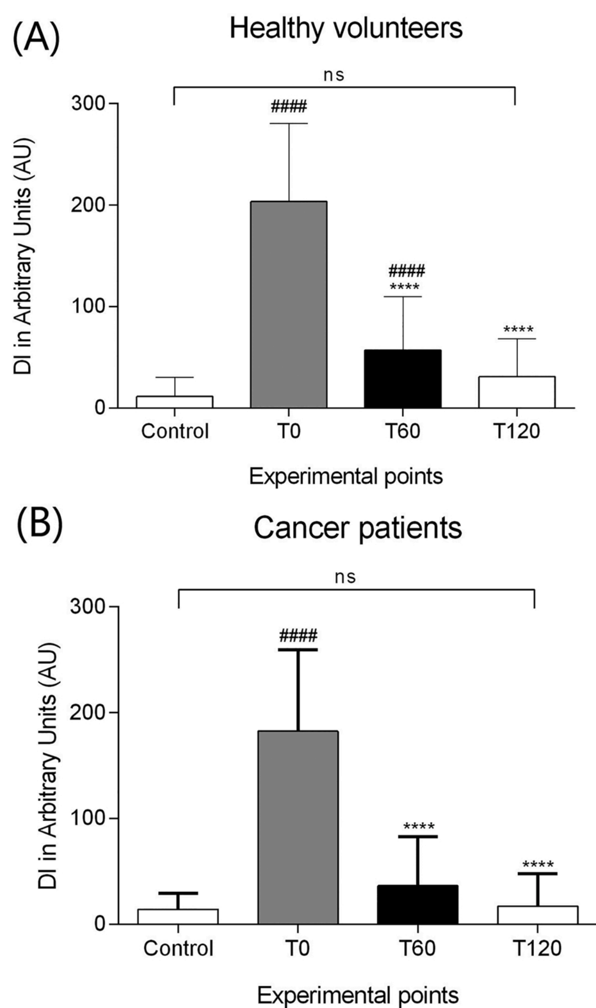

Fig. 1. Genotoxic damage index over time. (**A**) Healthy volunteers. (**B**) Patients without skin reaction. Peripheral lymphocyte genotoxic damage index (DI, expressed in arbitrary units) at different times: before irradiation (Control); immediately after irradiation (T0); 60 and 120 min after exposure (T60 and T120, respectively). Results are expressed as means ± SD. ^{ns} $P > 0.05$ T120 vs Control; $^{####}$ $P < 0.0001$ vs Control; **** $P < 0.0001$ vs T0; statistical analysis by Shapiro-Wilk test and Mann Whitney Wilcoxon test a posteriori.

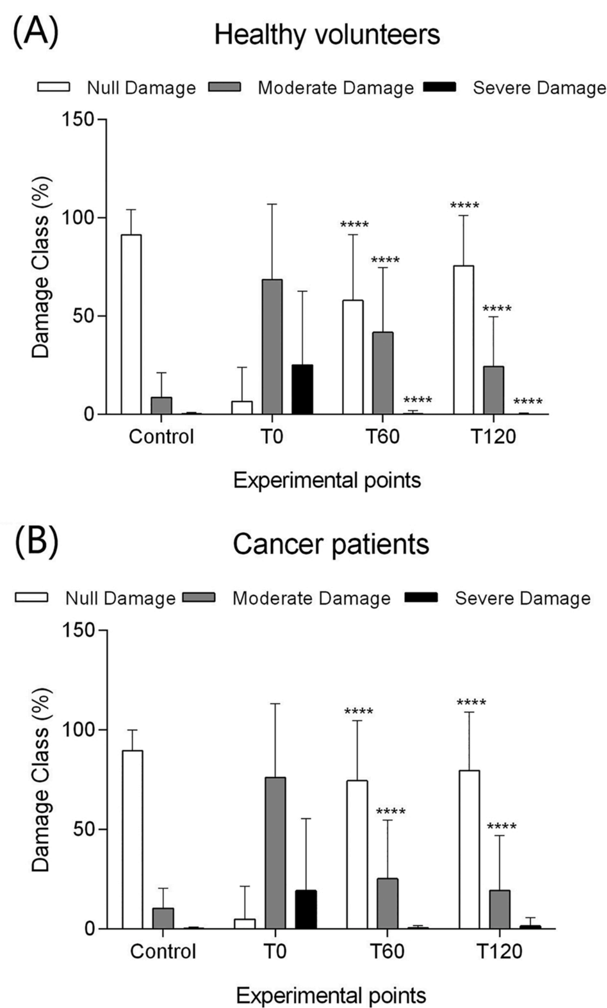

Fig. 2. Categorized damage over time. (**A**) Healthy volunteers. (**B**) Patients without skin reaction. Category of DNA damage for each analyzed point: before irradiation (control); immediately after irradiation (T0); 60 and 120 min after exposure (T60 and T120, respectively). Results are expressed as means ± SD. ****$P <$ 0.0001 vs the same class of damage at T0; statistical analysis by chi-square test.

was observed in BC patients without radiodermatitis, *i.e.*, DI increased from 14.2 ± 15.2 (SEM: 3.9) to 182.3 ± 76.9 (SEM: 19.9) after exposure ($P < 0.001$) and decreased 60 and 120 min post-irradiation by 80 and 90 %, respectively (Fig. 1B). Comparison of each analyzed point between healthy individuals and BC patients showed no statistically significant differences.

Changes in the frequencies of the different grades of DNA damage during repair were evaluated. In the healthy volunteers, most damaged cells after exposure (t = 0) showed moderate or severe damage (68 and 25 %, respectively; Fig. 2A). In BC patients without skin reaction, cells presented moderate (76 %) or severe (19 %) damage (Fig. 2B). After 60 and 120 min exposure to radiation, the percentage of cells with moderate or severe damage decreased drastically in both groups ($P < 0.001$; Fig. 2). Frequencies of cells with severe damage declined to values similar to those observed before irradiation.

From the DI data for healthy individuals and BC patients without radiodermatitis (grade 0 from the RTOG score), we constructed a DNA repair curve as a function of time (Fig. 3A). Both curves fit an exponential decay equation with the model $y = (y0 - Plateau)\ e^{(-Kx)} + Plateau$ and they are not significantly different. Three additional measurements, corresponding to three patients with radiotoxicity, are presented in Fig. 3A. Radiodermatitis grades and doses for these patients were as follows: patient 4: grade 3, 50 Gy; patient 13: grade 1, 21.6 Gy; patient 14: grade 3, 39.6 Gy.

Fig. 3B shows the DNA damage repair pattern obtained from the data set for healthy individuals, with the confidence intervals and the equation of the curve.

In summary, patients without skin reaction showed significant differences neither in initial DNA damage nor in radiation-induced damage, compared to healthy individuals. Similar DNA repair kinetics were observed between these groups, with no significant differences in residual genotoxic damage. In contrast, patients with acute side effects showed significantly lower DNA repair ability and significantly more residual damage compared to patients who did not develop radiation effects on the skin.

4. Discussion

Radiotherapy is a fundamental tool for the treatment of breast cancer, but individual RS is a critical problem that limits this treatment, affecting the quality of life of patients [2,7]. The factors determining RS are not fully understood [8,29,30] and it is important to determine them, as a basis for action [2,6]. Using the alkaline comet assay, one can

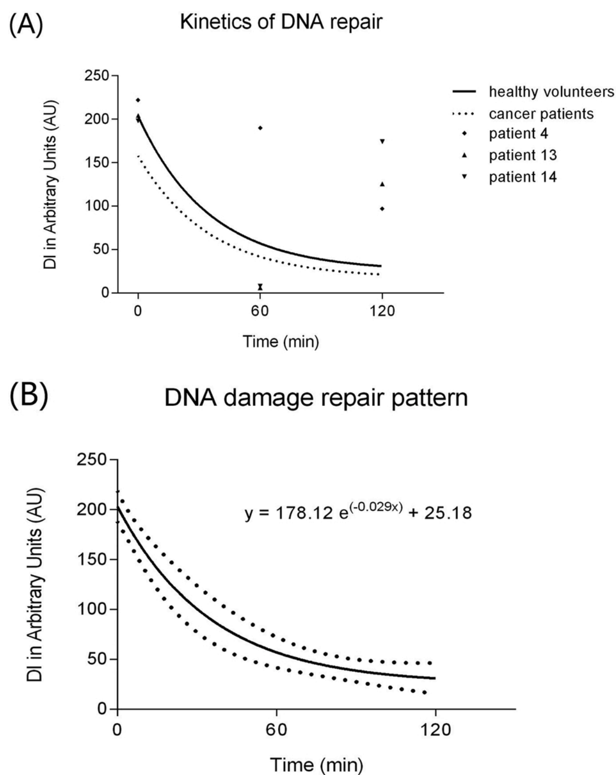

Fig. 3. Kinetics of DNA repair. (**A**) Mathematical fit of DI values over time to different curves for each group, healthy volunteers (full line) and patients without skin reaction (dotted line): immediately after irradiation (T0); 60 and 120 min after exposure (T60 and T120, respectively). Three other measurements are plotted, representing the behaviour of patients with radiodermatitis. (**B**) Mathematical fit of DI values over time for healthy volunteers (full line) and confidence intervals (dotted line).

detect induced genomic lesions and quantify their repair [9,12,24,31,32].

Patients with BC [31] and healthy individuals with a family history of cancer [33] have been reported to have lower DNA repair capacities. We prepared a standard curve for healthy individuals, to test whether individuals who deviated from the standard response developed radiotoxicity. We analyzed three parameters per individual: baseline genotoxic damage in non-irradiated cells, radio-induced genotoxic damage, and DNA repair capacity ($t = 120$ min). No significant differences were observed between healthy individuals and BC patients without radiotoxicity. Although greater genetic instability and/or induced persistent (nonrepaired) DNA damage might have been expected in cancer patients, we did not detect it [31]. Patients without radiotoxicity showed significant differences neither in baseline nor in radio-induced damage, compared to healthy individuals. Similar DNA repair kinetics were also observed in the two groups. This result is consistent with the hypothesis that neither chromatin structure nor antioxidant enzyme activities (fundamental in the establishment of the initial radiation-induced damage) contribute significantly to the clinical effects [34].

We acknowledge that discrepant results might occur due to factors such as the cohorts of healthy volunteers and patients (hereditary or spontaneous cancer) being analyzed, treatments prior to blood sampling, or irradiation protocols [31]. Also, it could be considered the interindividual difference attributed to some reparation systems such as the nucleotide excision repair (NER) pathway [33].

Three patients with acute effects [2] exhibited differential DNA repair capacity. Although one of the patients presented Grade 1 (mild) radiodermatitis that would generally not be considered clinically significant, this occurred early in the treatment, that is, at a low cumulative dose. Even though the three symptomatic patients did not start from a high radio-induced genotoxic damage, they showed significantly higher residual damage compared with the average patients, without skin reaction. According to Applegate et al. [6], deficient removal of lesions during fractional irradiation causes them to accumulate and can determine radio-toxicity and influence carcinogenesis. Therefore, this endophenotype might help to identify patients who are at increased risk of unacceptable response to radiotherapy. However, RS is a complex trait that is difficult to predict using a single technique; analysis of complementary pathways could provide valuable information.

5. Conclusions

Although many factors (e.g., dosimetry, irradiated volume, age, comorbidities, and lifestyle) may influence individual radiosensitivity and radiosusceptibility to carcinogenesis, evaluation of the repair of radiation-induced DNA lesions could help clinicians to predict adverse effects of radiotherapy. In pursuit of this goal, the use of bioassays to predict individual RS requires cross-validation and integration of multicenter cohort data.

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Declaration of Competing Interest

The authors report no conflict of interest.

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