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Evaluation of the *in vivo* genotoxic effects of gamma radiation on the peripheral blood leukocytes of head and neck cancer patients undergoing radiotherapy

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The present study aimed to evaluate the genotoxic effects of ionizing radiation on non-target cells of Head and Neck Squamous Cell Carcinoma (HNSCC) patients exposed to various cumulative doses of gamma rays during radiotherapy. The ten patients (P1–P10) were treated with cobalt 60 gamma radiation (External Beam Radiotherapy) for a period of five to six weeks with a daily fraction of 2 Gy for 5 days each week. The genotoxic effects of radiation (single strand breaks – SSBs) in these patients were analyzed using the alkaline single cell gel electrophoresis (SCGE) technique, with the Olive Tail Moment (OTM) as the critical parameter. A sample of each patient's peripheral blood before starting with radiotherapy (pre-therapy) served as the control, and blood collected at weekly time intervals during the course of the radiotherapy served as treated (10, 20, 30, 40, 50 and 60 Gy) samples. *In vivo* radiosensitivity of these patients, as indicated by SSB's after the cumulative radiation doses at the various times, was assessed using Student's *t*-test. Significant DNA damage relative to the individual patient's pre-therapy baseline data was observed in all patients. Inter-individual variation of the genotoxic effects was analyzed using two-way ANOVA. The correlation between doses for the means of smoker and non-smoker patients was calculated using the Pearson test. The results of this study may indicate the need to reduce the daily radiotherapy dose further to prevent genotoxic effects on non-target cells, thus improving safety. Furthermore, these results may indicate that the estimation of DNA damage following exposure to a gamma radiation, as measured by the comet assay in whole blood leukocytes, can be used to screen human populations for radiation-induced genetic damage at the molecular level.

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1. Introduction

Radiotherapy is the most important non-surgical modality for the curative treatment of cancer. In 2004, in the United States alone, nearly 1 million of the 1.4 million people who developed cancer were treated with radiation. Of the 10.1 million people diagnosed with cancer worldwide each year [1], approximately 50% require radiotherapy, 60% of whom are treated with curative intent. In general, approximately 50% of cancer patients receive radiation therapy for their disease management [2]. Radiotherapy is also highly cost effective, accounting for only 5% of the total cost of cancer treatment [3]. Ionizing radiation is one form of radiotherapy treatment of cancer. The most frequent type of radiotherapy treatment for HNSCC patients is external beam radiation with gamma rays. This therapeutic intervention is considered as a double-edged sword, with both benefits and risks, because it has been classified as a potent human carcinogen [4]. Radiation exposure causes DNA strand breakage, chromosomal aberrations, mutations and overall genetic instability [5]. Genetic integrity is maintained by an intricate network of DNA repair proteins [6]. Defects in this complex machinery are linked with familial predisposition to cancer and other diseases [7].

The aim of radiation therapy is to eliminate malignant cells while maintaining the integrity of the normal cells by employing an optimal dose of radiation. It is generally acknowledged that ionizing radiation kills mammalian cells by inducing damage to the nuclear DNA, although the ultimate cause of cell death in terms of DNA damage is controversial [8]. Several types of DNA damage and repair processes are induced by ionizing radiation. The sensitivity of both tumor cells and healthy tissues depends on the cell type and its proliferation and metabolic status [9,10]. The sensitivity also depends on intracellular scavenger concentrations and genetically determined factors [11–13].

Blood leukocytes are often employed to test genetically determined radiation sensitivity, mainly because they are readily available [14]. It is essential to study *in vivo* DNA damage in cancer patients who undergo radiotherapy to prevent or reduce the

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side effects of radiation exposure. Ionizing radiation is mutagenic and carcinogenic by virtue of its ability to damage DNA in cells, and thus, radiation therapy is also associated with an increased risk of incidence of secondary malignancies in cancer patients [15]. Hence, determination of radiation-induced DNA damage in humans has potential value for risk assessment. Monitoring of patients under radiotherapy for DNA damage could therefore contribute to the optimization of irradiation conditions and biological dosimetry. Peripheral white blood cells are often used as non-target cells for biological dosimetric studies. However, several studies have reported the use of whole blood rather than isolated lymphocytes for population studies [16]. Additional purification and culturing of the lymphocytes does not provide a definitive advantage because it is not clear at present whether any subtype would more closely approximate the effects on the target tissue [17]. We therefore evaluated whole blood in the present study.

The alkaline comet assay has become a popular technique for detecting a range of types of DNA damage during the last decade, and its usage in clinical practice has also increased rapidly [18,19]. Here, we compared the inter-individual differences in gamma radiation-induced damage of single strand breaks (SSBs) in peripheral blood leukocytes of HNSCC patients with different lifestyles. The DNA damage was determined by SCGE and subjected to statistical analysis.

2. Materials and methods

2.1. Subjects of study

Study participants with newly diagnosed HNSCC were recruited at Goa Medical College, Department of Radiation Oncology, Goa, India from a group of HNSCC patients who had not previously been treated with chemotherapy or radiotherapy. The population studied comprised 10 volunteer subjects (2 females and 8 males) diagnosed with SCC of the tongue, oropharynx, vocal cord and pyriform fossa. The patients gave their informed written consent and also provided information related to their lifestyles, such as their smoking status, medical history and exposure to chemical/physical agents, in responses on a specific questionnaire. Detailed patient data are provided in Table 1. Subsequent laboratory procedures involving the subjects and all investigations were carried out in accordance with a high standard of ethics under the guidelines of the Institutional Ethics Committee, Goa Medical College, Goa, India.

2.2. Radiotherapy

All patients underwent standardized external-beam partial-body irradiation with curative intent for localized tumors after a planned computed tomography scan and computer-generated distribution evaluation-assisted target localization and beam arrangement. An External Beam Radiotherapy Cobalt 60 source (Plate 1) was used for all patients, comprising lateral fields of the head and neck, with an average 1.25 MeV γ-ray beam of 2 Gy per day to the target area (Table 1). They received γ-radiation for five week(s) at a 10 Gy dose per week, up to a cumulative tumor dose of 54–66 Gy.

2.3. Sample collection and processing

Peripheral blood sampling was performed by venipuncture. Venous blood (5 ml) was collected in heparinized Vacutainer tubes (Becton Dickinson, NJ, USA) under sterile conditions. The peripheral blood of each patient collected prior to the initiation of radiotherapy was marked as his/her control sample. Seven samples were collected from each donor, one prior to the initiation of radiotherapy (control – 0 Gy) and the remaining six at weekly interval irradiations of 10 Gy for six weeks (treated). The pre-therapy blood sample (0 Gy) was collected on day 1 of the first radiotherapy cycle, 2 h prior to irradiation. The response of the peripheral blood leukocytes to the radiotherapy was evaluated in a blood sample collected within 1 h of the last dose of irradiation of the first week *i.e.* 10 Gy (after 5th day). Further blood samples were collected and handled in the same manner at various intervals of treatment *i.e.* after completion of the 20 Gy (2nd week), 30 Gy (3rd week), 40 Gy (4th week), 50 Gy (5th week) and 60 Gy (6th week) of irradiation. The blood samples, collected in vacutainer tubes, were coded and transported to the laboratory in an ice box. They were processed immediately (within a maximum of 1 h after collection) and subjected to further analysis by the alkaline single cell gel electrophoresis (SCGE) assay.

2.4. Single cell gel electrophoresis assay

The alkaline SCGE assay was performed according to the methods of Singh et al. [20] and Tice [21], with slight modifications. Fully frosted microscope slides (Fisher Scientific, cat no.: 12-544-5CY, USA) were coated with a thin layer of 1% normal melting-point agarose (LMA) and cooled to 4 °C. Subsequently, 20 μl of whole blood was mixed with 100 μl of 0.5% low melting agarose, and this suspension was pipetted onto the precoated slides and covered with a cover slip. The preparation was chilled for 5 min in the dark in a refrigerator at 4 °C, and after solidification of the suspension, the cover slip was removed.

2.4.1. Alkaline lysis

Slides with blood cells embedded in LMA were submersed in an alkaline cold (4 °C) lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base pH 10, 1% Triton X-100, 10% DMSO) at pH 10 and maintained at 4 °C for 4 h. They were then placed in an alkaline electrophoresis buffer of pH 13 (1 mM Na_2EDTA/300 mM NaOH) for 25 min to induce unwinding of DNA strands. The slides were then transferred to an electrophoresis tank with fresh alkaline electrophoresis buffer, and electrophoresis was performed at a field strength of 1.33 V/cm for 25 min at 4 °C (20 V/125 mA). Following electrophoresis, the samples were neutralized by incubation in 0.4 M Tris, pH 7.4, for 5 min.

2.4.2. Staining, microscopic analysis and experimental parameters

DNA was stained by placing 20 μl/ml ethidium bromide on the agarose, which was then covered with a cover slip and incubated for 5 min in the dark. From the time of placing the suspended cells on the slides through the electrophoresis, the cells were protected from additional DNA damage resulting from direct exposure to visible light by performing all steps in the dark at 4 °C. The DNA damage was visualized by observing the cells under 20× objective magnification of an epifluorescent microscope (Olympus BX 53, Japan) equipped with an excitation filter of 510–560 nm and an emission filter of 590 nm. One hundred comet images were recorded for each sample (2 slides, 50 images from each). The cells were analyzed by the image analysis software CASP [22]. OTM, which is the product of the percent tail DNA and the distance between the center of gravity of the head and tail, was used as a measure of DNA damage [23]. The OTM for each image was used as the variable of interest.

2.5. Statistical analysis

Intra-individual variation between the control and treated samples was analyzed using Student's *t*-test, and inter-individual variation was analyzed by two-way analysis of variance (ANOVA). The correlation coefficient (r^2) between the smokers and non-smokers was calculated using the Pearson test. The results are presented as the mean ± SD, and the values of $P < 0.001$, 0.01 and 0.05 were regarded as statistically significant.

3. Results

The DNA damage present in the peripheral leukocytes of the patients prior to radiotherapy and after various dose intervals of treatment (10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy and 60 Gy), measured as OTM values using the CASP software, is presented in Table 1. All the patients exhibited dose-dependent increases in DNA damage. The baseline (pre-therapy) DNA damage in peripheral leukocytes at 0 Gy (control) indicates inter-individual variation. The smoker (S) patients exhibited a high percentage of DNA damage (7 ± 2.2–16.4 ± 4.3), which was significantly higher than that of the non-smoker (NS) patients (0.1 ± 0.04–7.5 ± 2.5).

The OTM value at 10 Gy ranged from a minimum of 0.4 ± 0.2 (P2) to a maximum of 18.6 ± 8.2 (P7). The values for smokers after the 10 Gy dose ranged from 12.9 ± 4.9 to 18.6 ± 8.2 and were significantly greater than the values for non-smokers (0.4 ± 0.2–16 ± 2.5). The values of DNA damage at 20 Gy ranged from a minimum of 1.8 ± 0.7 (P1) to a maximum of 35.1 ± 16.1 (P6). All of the patients showed significant ($P < 0.001$) increases in DNA damage relative to their respective pre-therapy control values. The 20 Gy dose effects varied from 16.1 ± 2.5 (P5) to 35.1 ± 16.1 (P6) in smokers and from 1.8 ± 0.7 (P1) to 29.4 ± 17.9 (P10) in non-smokers.

In addition to the significant increase of DNA damage at 30 Gy in all patients relative to their respective pre-therapy values, elevated DNA damage was observed in smokers (24.8 ± 9.4–53.1 ± 15.4) compared with non-smokers (7.4 ± 2.6–52.3 ± 15.8). All patients showed a significant increase with respect to their individual control values at 40 Gy of irradiation. The OTM values for smokers

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