Correlation between DNA Repair Capacity in Lymphocytes and Acute Side Effects to Skin during Radiotherapy in Nasopharyngeal Cancer Patients

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Abstract

Purpose: Repair of radiation-induced DNA damage plays a critical role for both the susceptibility of patients to side effects after radiotherapy and their subsequent cancer risk. The study objective was to evaluate whether DNA repair data determined in vitro are correlated with the occurrence of acute side effects during radiotherapy.

Experimental Design: Nasopharyngeal cancer patients receiving radiation therapy were recruited in a prospective epidemiologic study. As an indicator for clinical radiosensitivity, adverse reactions of the skin were recorded. Cryopreserved lymphocytes from 100 study participants were γ-irradiated with 5 Gy in vitro and analyzed using the alkaline comet assay. Reproducibility of the assay was determined by repeated analysis (n = 22) of cells from a healthy donor. A coefficient of variation of 0.24 was calculated.

Results: The various parameters determined to characterize an individual DNA repair capacity showed large differences between patients. Twenty-one patients were identified with considerably enhanced DNA damage induction, and 19 patients showed severely reduced DNA repair capacity after 15 and 30 minutes. Eight patients were considered as clinically radiosensitive, indicated by moist desquamation of the skin after a total radiation dose of 70 Gy.

Conclusions: Using the alkaline comet assay as described here, nasopharyngeal cancer patients were identified showing abnormal cellular radiation effects, but this repair deficiency corresponded only at a very limited extent to the acute radiation sensitivity of the skin.

Despite various therapeutic improvements, efficient radiation therapy of malignant cancers is limited by the adverse side effects occurring in the normal tissue when exposed to radiation. In general, >15% of nasopharyngeal cancer patients develop acute or late symptoms of enhanced radiosensitivity (1, 2). Several patient- and treatment-related factors are known to influence the variability of side effects; however, up to 70% of the cases remain unexplained (1). Therefore, there is much interest among clinicians for in vitro detection of cellular radiosensitivity as an indicator of the extent of a patient’s normal tissue reaction (3). The use of such predictive assays would enable clinicians to adjust radiation therapy for both sensitive and resistant patients (3) with consequent improvement in the therapeutic ratio (5). For example, a strategy based on testing human normal tissue radiosensitivity to identify the patients with a high risk of developing unacceptable severe reactions after radiotherapy might also permit the individualization of treatment (5) by dose escalation in resistant patients without increasing normal tissue complications (6–8).

Three biological parameters have been used mainly to determine radiosensitivity in vitro. Data from assays based on clonogenic survival (9–12) and chromosomal aberrations (11, 12) showed, at least in several studies, a good correlation of in vitro data with late radiation effects, but these assays are time consuming and not very suitable for the characterization of a large number of patients. The nature of the genetic defects associated with radiosensitivity point out that reduced cellular DNA repair capabilities may play a major role in causing radiosensitivity (13–16). Thus, the characterization of DNA repair in lymphocytes might be a suitable approach to predict clinical radiation reactions.

In particular, the alkaline single-cell microgel electrophoresis assay (or comet assay) has been shown to be useful for the assessment of DNA damage and repair within epidemiologic studies, because it is a fast and reliable assay that needs only a small number of cells (17–19). Using the comet assay, enhanced initial damage and reduced repair capacity in vitro were characterized for ataxia telangiectasia patients (20, 21). Furthermore, significant differences in DNA repair kinetics were detected between patients with severe skin reactions and patients with normal reactions to radiation when the alkaline...
comet assay was used (13, 22). However, these studies suffer from several weaknesses, among them the retrospective case-control study design with unclear selection of participants and insufficient characterization of therapy modalities and side effect documentation (23).

Therefore, we recruited in a prospective study nasopharyngeal cancer patients receiving radiation therapy. Acute side effects of the skin were used as an indicator for clinical radiation sensitivity, and radiation-induced DNA damage and repair were determined with the alkaline comet assay in peripheral blood lymphocytes. The correspondence of clinical radiosensitivity and in vitro data were studied to evaluate whether side effects can be predicted by the assay.

Materials and Methods

Study subjects. An unselected cohort of 216 nasopharyngeal cancer patients was recruited at three radiotherapy center in Xinqiao Hospital, Southwest Hospital, and Chongqing Tumor Hospital. Patients who had been treated with chemotherapy were excluded. The study was approved by the Ethical Committee of the Medical Faculty at the Third Military Medical University. Patients gave informed written consent. Information on the tumor, its therapy, and the side effects of radiation therapy was gathered from patient records, and personal information was collected using self-administered patient questionnaires. Patient recruitment started in June 2000.

For logistic reasons, radiation-induced DNA repair was determined in 133 patients ascertainment until December 2001. The age of these 133 patients ranged from 32 to 70 years, with a mean age of 58 years. All patients received a total dose of 70 Gy. The acute side effects of radiotherapy developing in the skin within the radiation field were used as an indicator for clinical radiosensitivity. They were documented at three time points before, during, and after radiotherapy. Assessment of side effects of the skin followed a modified classification system based on the Common Toxicity Criteria of the NIH (24). Development of side effects of at least grade 2 was considered as an enhanced radiation reaction and was, therefore, taken as an indicator for acute clinical radiosensitivity. For this analysis, only skin reactions that occurred by the third observation date were considered to avoid differences resulting from variations in the maximum radiation dose received.

Lymphocyte isolation and treatment of cells with phytohemagglutinin. Before radiation treatment, a venous blood sample of 80 mL was obtained in a citrate-layered monovette for each patient. For control experiments, a buffy coat from a 400 mL blood donation of a healthy individual was received from the blood bank of the Xinqiao Hospital. Lymphocytes were obtained through gradient centrifugation by overlaying whole blood or buffy coat on Lymphoprep (Nycomed Pharma, Asker, Norway) in Leucosep tubes (Sarstedt, Nümbrecht, Germany), centrifugation at 800 g for 15 minutes at room temperature, removal of lymphocyte-containing layers, and two washing steps with PBS (Life Technologies, Wetzlar, Germany). Aliquots of 2 × 10^5 lymphocytes were suspended in cryomedium [RPMI 1640 (Life Technologies) containing 50% of heat-inactivated FCS and 10% DMSO], cooled to −80°C at a rate of −1°C/min in a freezing container (Neolab, Frickenhausen, Germany), and stored in liquid nitrogen.

Before comet assay experiments, the frozen cells were rapidly thawed, washed, and resuspended at a concentration of 1 × 10^6 cells in RPMI 1640 supplemented with 2 mMol/L-glutamine, 10% FCS, 10 mMol/L HEpes buffer, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2% phytohemagglutinin (all supplements were from Life Technologies) for mitogenic stimulation. Cell suspensions were cultured for 20 hours at 37°C and 5% CO2.

Alkaline single-cell microgel electrophoresis assay. Before γ-irradiation, each lymphocyte sample was divided into four aliquots at a density of 1 × 10^5 cells per 500 µL RPMI medium and cooled on ice. Three of the aliquots were irradiated with 5 Gy using a 137Cs source (Gammacell 1000, Atomic Energy of Canada, Ltd., Edmonton, Canada) at a dose rate of 12 Gy/min. After treatment with ionizing radiation, two samples were kept at 37°C for 15 and 30 minutes to allow for DNA repair before being subjected to the comet assay. The third sample and the nonirradiated control cells were kept on ice and processed directly after the irradiation procedure. To determine experimental variability, lymphocytes of the healthy control individual were treated similarly as were patients’ samples. In total, analysis of reference lymphocytes was repeated 22-fold.

The comet assay was done basically as described (18–20) with some modifications. Briefly, a total of 50 µL suspension (1 × 10^6 cells) were mixed with 350 µL of low-melting agarose (0.7%). After incubation with a lysis solution [0.3 M NaCl, 10 mM Tris (pH 10.0)], the samples were subjected to electrophoresis (25 V and 300 mA for 20 minutes). SYBR Green staining (Molecular Probes, Amsterdam, the Netherlands), and observation under a Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The nuclei were classified into four groups based on the length and fluorescence intensity of the comet tail: type 1, no tail (true nucleus); type 2, comet with short tail (tail length less than the head diameter); type 3, comet with tail length longer than the head diameter and with high fluorescence intensity; type 4, comet with tail length longer than the head diameter and with low fluorescence intensity. Types 1 to 4 were weighted by special factors of 0 to 3, respectively, and comet scores were calculated as 0 × n_1 + 1 × n_2 + 2 × n_3 + 3 × n_4, where n is the number of cell with comet types per 100 counted cells.

For all 133 blood samples, the assay could not be evaluated because of the low number of viable cells recovered from the frozen blood sample. Another two patients had to be excluded from the analysis because they did not meet the eligibility criteria of the study. Thus, samples and data from 100 patients could be used for analysis.

Lymphocytes of a healthy individual were used as a reference sample and were irradiated and treated in parallel with the patients’ samples at several experimental dates. Thus, results from 22 independent experiments were obtained for the reference sample. These data were used to calculate experimental variability and the cut points of the assay parameters to identify individuals with abnormal reactions toward radiation effects.

Statistical methods. The correlation between the three assay parameters was tested using the Spearman rank correlation coefficient. Analysis was done with Statistical Analyses System software (SAS, Release 8.2; SAS Institute, Inc., 1999, Cary, NC). Figures were drawn with SigmaPlot 2001 for Windows, version 7.0.

Results

Dose selection for the determination of cellular radiation effects with the comet assay. First, dose-response curves at various time points after irradiation were established using lymphocytes of a healthy donor to determine optimal conditions (Fig. 1). Cells were irradiated with 0, 2.5, 5, and 10 Gy. To quantify the DNA damage induced (i.e., DNA strand breaks and alkaline labile sites), tail moments were measured after alkaline electrophoresis using an automatic evaluation system (19). Tail moments determined immediately (at 0 minute) after irradiation increased with the radiation dose applied (Fig. 1) and represented the DNA damage that had been present initially and impeded a fast evaluation of this data point. After a time interval of 15 minutes, the DNA damage detected was reduced because of DNA repair, and 30 minutes after irradiation ~70% of the DNA damage that had been present initially were removed, indicating that DNA repair was rapid but not complete at this time point.
Fig. 1. Determination of DNA damage induced by γ-irradiation with the alkaline single-cell electrophoresis assay. Peripheral blood cells of a normal donor were irradiated with increasing doses of γ-rays and the DNA damage induced was measured as the tail moment at three different time points (0, 15, and 30 minutes after irradiation). Each point of the dose-response curves was determined on three independent slide areas evaluating 160 cells in total. Bars, SD.

Based on this dose-response curve, and as only a limited amount of lymphocytes from patients was available, we decided to irradiate lymphocytes of nasopharyngeal cancer patients with one single irradiation dose of 5 Gy and to measure the radiation-induced DNA damage after 0, 15, and 30 minutes. To determine the background damage, a sample of nonirradiated, sham-exposed cells was also included.

**Induction of DNA damage and DNA repair capacity in γ-irradiated lymphocytes from nasopharyngeal cancer patients.** Induction and repair of DNA damage after γ-irradiation were determined in lymphocytes from 100 nasopharyngeal cancer patients. Five experimental parameters were acquired: (a) baseline DNA damage detectable in cells that had not been irradiated, (b) DNA damage measured directly after irradiation, (c) DNA repair capacity after 15 minutes that was defined as: \[1 - \frac{\text{mean tail moment at 15 minutes}}{\text{mean tail moment at 0 minute after irradiation}}\], (d) DNA repair capacity after 30 minutes that was calculated using the mean tail moment measured after 30 minutes, and (e) the DNA repair capacity between 15 and 30 minutes calculated as: \[1 - \frac{\text{mean tail moment at 30 minutes}}{\text{mean tail moment at 15 minutes after irradiation}}\]. Background damage, DNA damage induced, and DNA repair capacity after 30 minutes are summarized for all patients as box plots in Figs. 2 and 3. The figures present the range of assay parameters observed in the study subjects, as well as the medians and the 25th, 75th, and 70th percentiles. In addition, the results for lymphocytes from the healthy blood donor are shown that were included in 22 different electrophoresis runs as reference samples. The data characterizing the DNA repair capacity after 15 minutes were processed accordingly.

The three experimental parameters presented in Figs. 2 and 3 showed nearly identical mean (solid lines) and median values (dotted lines) for the reference sample and the patients. In addition, a very similar range of distribution was found for the results of the 22 reference experiments and of the patients and is marked by the boundaries of the boxes that represent the 25th and 75th percentiles. Patients with results lying within the 25% to 75% range of the internal standard were considered to show a “normal” cellular reaction to γ-irradiation. In addition, the patients exhibiting less damage in nonirradiated or irradiated cells [Fig. 2] or higher DNA repair capacity (Fig. 3) than marked by the 25% to 75% range were also classified as normal.

Regarding the relationship between the experimental parameters measured, data analysis revealed that there was a significant correlation between the DNA damage present in nonirradiated cells and the DNA damage induced (\(r = 0.68, P = 0.0001\)) and a weak but significant correlation between DNA damage induced and DNA repair capacity after 30 minutes (\(r = 0.28, P = 0.0032\)). DNA damage present in nonirradiated cells and DNA repair capacity after 30 minutes were not correlated.

**Correlation of cellular radiation effects determined in vitro and clinical radiosensitivity characterized by skin reactions.** The comparison between clinical signs of radiosensitivity and repair parameters determined in vitro as indicators of cellular radiosensitivity is summarized in Table 1. It indicates that there is no apparent correlation between the acute skin reactions to radiation occurring in vivo and the in vitro radiation effects in lymphocytes as they were determined in this study. When the DNA damage induced by 5 Gy of irradiation was evaluated as an experimental marker for clinical radiosensitivity, three of the eight reactive patients showed enhanced or high damage. Similarly, three of the eight patients with grade 2C reactions to radiation therapy exhibited a reduced DNA repair capacity in the time interval between 15 and 30 minutes.

**Discussion**

Because an intact DNA template is a fundamental prerequisite for normal cellular function, our hypothesis is that DNA
repair plays an essential role in avoiding cellular reactions after irradiation and that efficient DNA repair will reduce radiation sensitivity. We measured g-radiation–induced DNA damage and repair in peripheral blood lymphocytes from 100 nasopharyngeal cancer patients for whom, within a prospective epidemiologic study, clinical radiation sensitivity was evaluated on the basis of acute skin reactions developing during radiation therapy. We identified 21 patients exhibiting enhanced initial radiation damage and 19 patients with reduced DNA repair capacity after both 15 and 30 minutes. According to our hypothesis, these patients should exhibit enhanced clinical radiation sensitivity. However, the comparison with the clinical data revealed that only three of the patients suffered from increased acute radiation reactions of the skin.

After γ-irradiation, different types of lesions can be detected in the nuclear DNA. DNA single- and double-strand breaks and a plethora of modified nucleotides are induced by direct ionization of the DNA and by free radical–mediated reactive oxidative species developed by radiolysis of water (26, 27). In addition, clusters of DNA damage are produced by ionizing radiation as a consequence of the nonuniform deposition of ionizing radiation energy (26). The comet assay used under alkaline conditions is able to detect a major part of these radiation-induced lesions such as single-strand breaks, alkali-labile sites, and double-strand breaks. Double-strand breaks and their repair are regarded by many authors to be critical lesions for cell survival.

In summary, the alkaline comet assay was designed to provide the most comprehensive picture of DNA damage induced. In addition, the assay can quantify the cellular capacity to repair the observed lesions in showing disappearance of damaged sites and restoration of the genome. A limitation for using the assay might be that it does not provide information about the fidelity of DNA lesions repaired. DNA damage sensitive to repair and free radicals might indicate impaired DNA repair capability (28). However, assays to determine fidelity of DNA repair are difficult to use in epidemiologic studies.

For the comet analysis, peripheral blood lymphocytes were used that are easily obtained from patients but that are not the target cells developing adverse reactions in the irradiated normal nasopharyngeal tissue during therapy (8, 29). However, because of the genetic background of a possible repair defect, radiosensitivity as a consequence of impaired DNA repair should also be measurable in surrogate cells such as lymphocytes (7, 12).

To determine the reproducibility of the assay, lymphocytes of a healthy blood donor were analyzed in 22 independent experiments. These data were used to calculate the experimental variability, which was rather high (30%).

From the comet analysis, five experimental markers were derived for each patient: background DNA damage in nonirradiated cells, radiation-induced DNA damage, and DNA repair capacity after 15 and 30 minutes. The late repair interval between 15 and 30 minutes was evaluated separately as a fifth marker. Similar markers were analyzed in previous studies using the comet assay or other methods to detect DNA damage (13, 16, 22).

The DNA damage induced directly after irradiation is considered by several authors to be responsible for clinical radiation sensitivity, because a large amount of DNA damage will be harmful to the cell. The DNA damage induced by a radiation dose has been shown to be up to 3-fold higher in a radiation-sensitive mouse lymphoma cell line than in the parent cell line (30). Similar to our experiments, others have found this parameter to differ considerably between individuals; although the applied dose was constant (13–16, 22). An explanation might be that the effective dose reaching the target DNA is modulated by the genetically determined cellular content of radical scavenging enzymes and

**Table 1. In vitro repair data for γ-radiation–induced DNA damage and development of skin reactions of score 2c in nasopharyngeal cancer patients during radiation therapy**

<table>
<thead>
<tr>
<th>Skin reactions</th>
<th>DNA repair capacity</th>
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<td>15 min</td>
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<td></td>
<td>Normal</td>
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<tr>
<td>≥2c</td>
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<td>&lt;2c</td>
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metabolites (31, 32). In addition, mutations in DNA repair genes can affect the initial DNA damage induced by a constant irradiation dose, which was found to be enhanced in ataxia telangiectasia patients and in ataxia telangiectasia heterozygous individuals (20). Similarly, genetic variants in base excision repair genes can affect the amount of DNA damage induced by irradiation (33). It was suggested that the variability detected with the comet technique in the initial damage of cells with different genetic background might point at a difference in the DNA attachment in the nuclear matrix or in chromatin density (21). In most of the clinical studies cited previously, the initial DNA damage was shown to correlate with radiation sensitivity, mainly when late effects of a therapy were evaluated. In a study where acute effects were analyzed separately from late effects, such a correlation was not found (22).

The third experimental parameter, DNA repair capacity, characterizes the cellular ability to remove DNA damage and to restore the intact genome. Most authors evaluated the DNA repair capacity after irradiation (13–16, 22) or the amount of residual DNA damage after a repair interval (15, 29). As a result, an influence of impaired DNA repair on late radiation reactions, mainly fibrosis, was frequently found.

Our data do not support a correlation between reduced DNA repair capacity and acute clinical radiation effects of the skin. It should be noted here that repair kinetics are expected to be quite different for various types of lesions. An example are single-strand breaks that are repaired within a few minutes after irradiation, whereas double-strand breaks can require >2 hours for repair (26). That three patients with grade 2C reactions of the skin shown reduced DNA repair capacity at the late repair interval between 15 and 30 minutes might indicate that observation of DNA lesions with “slow repair kinetics” would be more useful in explaining clinical symptoms than observation of DNA lesions with “fast repair kinetics” (such as single-strand breaks and alkali labile sites).

Generally, the radiation damage to normal tissue was due to the amount of DNA damage. The work presented here suggests that irradiation-induced damage may be due not only to DNA damage, but also to other cellular responses to the irradiation. Therefore, to better predict the acute radiation sensitivity of normal tissue in cancer patients, further study is needed to elucidate the mechanisms of radiation injury in cells.

In conclusion, the alkaline comet assay as used in this study can identify individuals with a DNA repair defect, but the repair deficiency measured corresponded only at a very limited extent to the occurrence of acute radiation sensitivity of the skin in nasopharyngeal cancer patients. The suitability of the assay to predict late adverse reactions will be analyzed in the future. In addition, it is suggested that a more detailed analysis of repair kinetics optimized for different radiation-induced DNA lesions and an analysis of the fidelity of DNA repair might be more informative for predicting side effects of radiation therapy.

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DNA Repair and Normal Tissue Reaction in Radiotherapy

RETRACTED, April 21, 2009
Retraction: Correlation between DNA Repair Capacity in Lymphocytes and Acute Side Effects to Skin during Radiotherapy in Nasopharyngeal Cancer Patients

To the Editor: The authors withdraw the article titled, “Correlation between DNA repair capacity in lymphocytes and acute side effects to skin during radiotherapy in nasopharyngeal cancer patients,” which appeared in the July 15, 2005, issue of Clinical Cancer Research (1). The first author reproduced sections without quotations or appropriate citation from the article titled, “Radiation-induced DNA damage and repair in lymphocytes from breast cancer patients and their correlation with acute skin reactions to radiotherapy” (O. Popanda, R. Ebberler, D. Twardella, et al. Int. J Radiat Oncol Biol Phys 2004 Apr 1; 55 (5):1216–25; ref. 2). Although our research was on nasopharyngeal cancer rather than on breast cancer as published by the authors of the International Journal of Radiation Oncology*Biology*Physics article and contained some different data, reproducing the work of Popanda and co-authors and presenting it as our own, cause us to retract the article. The authors regret our actions and apologize to the scientific community and to Clinical Cancer Research for breaching scientific practices and editorial policies.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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