Estimating DNA Repair by Sequential Evaluation of Head and Neck Tumor Radiation Sensitivity Using the Comet Assay

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Background: The alkaline comet assay is a microelectrophoretic technique for detecting single-strand DNA breaks, and may be used as an indirect measure of hypoxia by determining the radiation sensitivity of individual cells.

Objective: To assess the ability of the comet assay to estimate the rate of DNA repair after irradiation in patients with head and neck cancer.

Methods: The comet assay was used to evaluate DNA damage in fine-needle aspirates of lymph nodes containing metastatic squamous cell carcinoma in patients with head and neck cancer 1, 2, and 3 minutes after treatment with 500 rad (5 Gy) of irradiation. The amount of DNA damage (measured as the “tail moment” of the comet) is proportional to the number of DNA single-strand breaks after irradiation, which in turn depends on the oxygen concentration in each cell.

Results: The mean ± SD of the median tail moment of the 1-minute postirradiation comets was 29.4 ± 14.2 (n=27). After 2 minutes, the mean median tail moment decreased to 25.4 ± 13.6 (n=25), representing a mean decrease of 11.9% in those patients with both 1- and 2-minute comet assays. Assuming a linear rate of repair, this decrease in DNA damage corresponds to a repair half-life of 4.2 minutes. A 3-minute assay was also performed on samples from a smaller number of patients (n=9), with a mean value not significantly different from that of the 2-minute assay of the samples from this group.

Conclusions: The comet assay is a promising tool for evaluating radiation sensitivity in individual cells. The rate of DNA repair early after irradiation is consistent with data in the literature.


Hypoxia in head and neck tumors limits the efficacy of conventional radiotherapy.1-4 The success of radiotherapy requires sufficient cellular oxygen, because irradiation induces damage and apoptosis by converting oxygen into reactive oxygen species, which in turn breaks DNA strands. Olive5 confirmed that tumor cells from mice breathing air during irradiation suffer more DNA damage than cells from mice asphyxiated before exposure. Since strategies for overcoming tumor hypoxia are becoming available, the assessment of tumor oxygenation is important because it may allow for customization of nonsurgical treatment.

Several techniques have been used to evaluate hypoxia in solid tumors, including the oxygen microelectrode assay, the paired survival assay, and the comet assay. The oxygen microelectrode, considered the “gold standard” in measuring tumor oxygenation, has been shown by some authors to correlate with the effect of radiotherapy.6,7 However, this technique does not detect radiobiological hypoxic fractions. The paired survival assay provides information on the hypoxic fraction of tumor cells.2,8 However, it requires removal of tumor cells and therefore would not be feasible for many human subjects. These disadvantages are overcome with the comet assay, which provides the ability to calculate a hypoxic fraction from a small sample (fine-needle aspirate) of cells.

The comet assay, also known as single-cell gel electrophoresis, was first applied to the study of hypoxia in cells by Olive et al.9,10 During this assay, tumors are exposed to 5 to 1500 rad (5-15 Gy) of radiation; then, cells are aspirated using a fine needle (22 g or smaller), subjected to electrophoresis, and viewed under a microscope. The DNA that has been damaged by irradiation travels further down the gel, producing a comet-shaped image with an illuminating head and a tail that lengthens in proportion to the severity of damage.
PATIENTS AND METHODS

PATIENTS

Twenty-seven patients with lymph node metastases from advanced, resectable head and neck cancer underwent a comet assay as a part of a larger randomized, clinical trial investigating the importance of hypoxia in radiation response and the efficacy of tirapazamine (a hypoxic cell cytotoxin). The patients ranged in age from 40 to 74 years, with a mean ± SD of 57.9 ± 8.7 years. There were 25 men and 2 women. An FNA was taken from the lymph node before any treatment (baseline study), and during the same encounter the node was treated with 500 rad (5 Gy) of radiation. Additional FNAs were taken from the same node 1, 2, and 3 minutes after the radiation treatment.

COMET ASSAY

The cells from each FNA were added to ice-cold saline and taken immediately to the laboratory. They were diluted in cold phosphate-buffered saline at a concentration of 2.4 × 10⁶ cells/mL; then, 300 µL of cell suspension was added to 1.5 mL of 1% low-melting agarose containing 2% dimethyl sulfoxide, mixed briefly, and spread on a microscope slide placed on a cold block. The agarose was allowed to gel and then placed in an alkaline lysis solution (30 mM sodium hydroxide, 1 M sodium chloride, and 0.1% N-lauroylsarcosine) for 1 hour. Before electrophoresis, the slides were rinsed thoroughly in alkaline rinse solution to remove residual sodium chloride, which inhibits DNA migration during electrophoresis. The slides were then placed in a horizontal-bed electrophoresis chamber containing 1.8 L of alkaline rinse solution and electrophoresed at 0.6 V/cm for 22 minutes. After electrophoresis, the slides were rinsed in distilled water, stained with propidium iodide (2.5 µg/mL in distilled water) to allow visualization of the DNA, and rinsed again to remove unbound stain.

Slides were analyzed by the method described by Olive and Durand. The DNA from individual cells was visualized using a microscope (Optiphot; Tokyo, Japan) with an epifluorescent attachment. Images for analysis were acquired using an image intensifier (Nitemate; Intevac, San Jose, Calif) coupled with a digital camera (4612 CCD; Ikegami, Tokyo, Japan) and digitized using a modular frame grabber (Imaging Technologies, Chicago, Ill). The image analysis was performed with software that calculates relative DNA content and tail moment for individual cells. DNA content is defined as the amount of fluorescence a comet image emits, and a tail moment is defined as the product of the percentage of DNA in the tail and the displacement between the center of the head and the center mass of the tail (Figure 1). Three hundred cells were analyzed from each sample when available; if fewer than 50 cells were available, the comet was considered unanalyzable. The 3-minute assay was added to the protocol after the 14th patient was enrolled.

DATA ANALYSIS

The median tail moment (MTM) from these assays was used to assess radiation sensitivity in the patients. A baseline MTM for each patient was also assessed using tumor cells that were not exposed to radiation. The baseline means and SDs were used to produce a baseline range, the minimum being the mean minus the baseline, and the maximum being the mean plus the baseline. All data points that fell within the baseline range were assumed to be unirradiated and were thus excluded. A paired, 2-tailed t-test was used to analyze DNA repair rates between 1 and 2 minutes and between 2 and 3 minutes. A Pearson correlation coefficient was used to evaluate the relationship between the patients’ age and the rate of DNA repair.

RESULTS

An example of a microscopic image of comets seen in normal cells and cells irradiated to 500 rad (5 Gy) is shown in Figure 2. The round head (Figure 2A) represents the cell, and the tail (Figure 2B) is composed of traces of DNA migration, which are reflections of single-strand DNA breaks. Figure 3 illustrates representative tail moment frequencies from a single patient for each time interval of 1, 2, and 3 minutes after baseline measurements have been excluded.

Very little DNA damage was seen in the baseline comet assays; the mean ± SD of the MTMs was 4.2 ± 3.9 (range, 0.7-20.2). The data for the baseline and the 1-, 2-, and 3-minute comet assays are depicted in Figure 4, which shows the MTM of each period. The mean of the MTM of the 1-minute irradiation comets was 29.4 ± 14.2 (n = 27). After 2 minutes, the mean MTM decreased to 25.4 ± 13.6 (n = 25). Twenty of the FNAs from these patients were subjected to both 1- and 2-minute comet assays, and the MTM in these 2 groups produced a mean decrease of 11.9%. Assuming a linear rate of repair, this corresponds to a repair half-life of 4.2 minutes. Samples

the DNA damage. The basis for this analysis is that ionizing radiation produces approximately 3 to 4 times fewer DNA single-strand breaks in anaerobic than in aerobic cells. It is therefore possible to use the amount of DNA damage produced to estimate the degree of hypoxia at the time of irradiation.

Many studies have shown that DNA damage caused by irradiation is reversible and can be restored to preirradiation levels within 30 minutes to 2 hours after exposure of up to 1500 rad (15 Gy). There is also evidence that irradiation in vivo produces half as much damage as irradiation in vitro, suggesting that there are rapid single-strand break-rejoining mechanisms that are able to repair DNA immediately after irradiation. There seems to be no significant difference in the DNA repair rate between tumor and normal cells or between aerobic and hypoxic cells.

Prior studies have explored the DNA repair competency within a span of 2 to 3 hours after irradiation, but little is known about this process immediately after exposure to radiation. A shorter, more defined time span of 1 to 3 minutes after exposure to 500 rad (5 Gy) of radiation was selected for evaluation in the current study, and DNA repair rates were compared.
from a small number of patients (n=9) also underwent a 3-minute comet assay, with a mean of 22.4±7.5, which was not significantly different from that of the 2-minute assay. The t test values indicated that the difference between the MTMs after 1 and 2 minutes was statistically significant (P=.02); the large mean decrease of 11.9% suggests that this is clinically significant as well. The change from the 2- to the 3-minute assay failed to reach statistical significance (P>.10).

No significant correlation between age and rate of DNA repair was observed, with a correlation coefficient of 0.30, which failed to reach statistical significance (P>.10).

**COMMENT**

The comet assay has proved to be a useful way of quantifying tumor hypoxia on a cellular basis. In the current analysis, we observed a substantial amount of DNA damage, which is measurable as an MTM. Also, we observed a statistically significant decrease in DNA damage when we compared the assay 2 minutes after irradiation with the assay 1 minute after irradiation. This difference most likely reflects DNA repair during the interval, but other potential explanations exist.

It is possible that the decrease in MTM may be a result of dilution by circulating unirradiated cells, particularly lymphocytes that may have migrated into the tumor. Also, we used serial sampling of cells, and it is possible that the first aspiration affected the subsequent aspirations in some way, eg, by inducing hemorrhage. However, measures were taken to minimize the influence of unirradiated cells by subtracting any comets that fell within a defined range, determined by the baseline study. Another precaution that was taken was to treat the cells with dimethyl sulfoxide to minimize the influence of red blood cell contamination of the samples. A less likely cause for the difference in MTM from one sample to the next is underlying variability among the cells sampled. Solid tumors are known to be heterogeneous, and it is possible that a sampling mismatch between the first and second comet assays led to detectable differences in the MTMs. The statistically significant difference between the samples, however, supports a nonrandom decrease in DNA damage. Furthermore, the reproducibility of the assay with multiple samples was investigated by Olive et al,13 who obtained 3 separate FNAs from 10 human tumors and found a high rate of concordance with the results obtained from a single FNA.

The comet assay remains a promising tool for evaluating radiation sensitivity in individual cells. An important advantage of this assay is its ability to detect DNA damage on a single-cell level without the necessity for radiolabeling. Because individual cells are observed, measurements from the comet assay are largely unaffected by cell type, DNA content, or necrosis.12,14 It is therefore possible to obtain an estimate of the oxygenation within a tumor.13 Furthermore, the small sample size necessary for analysis allows the use of FNAs. Numerous studies have confirmed that the comet assay provides an accurate estimate of the radiobiological hypoxic fraction over a wide range of tumor oxygenation, as it compared favorably with hypoxic fractions measured using the oxygen microelectrode assay,14 radiobiological assays,8 and the conventional paired survival curve assay.2,9,15,16 By identifying patients whose tumors are hypoxic before radiotherapy and determining the extent to which this hypoxia will impair tumor control, it may become possible to logically stratify patients to methods to overcome hypoxia before therapy.

In addition to estimating radiation resistance, the comet assay may prove to be valuable in evaluating the general aggressiveness of tumors. Hockel et al17 found that hypoxia may be a potential marker for both radiation resistance and aggressiveness of tumors, because pa-
patients with hypoxic tumors respond poorly to surgery as well as to radiotherapy. Since the comet assay provides measurements of tumor hypoxia, it may be extremely useful in estimating the disposition of the tumor as well as the relative success of therapy.

A significant disadvantage of using the comet assay is its technical requirement of a rather high radiation dosage in order to generate distinguishable responses of hypoxic and nonhypoxic cells. Often, this means a radiation dose of 350 to 500 rad (3.5-5.0 Gy), which is substantially more than the intensity usually given in the course of fractionated radiation therapy. Because of this technical requirement, the comet assay has been used in a limited way in human tumors. However, evidence of the rapid DNA repair shown in our study provides reassurance that the comet assay may be repeated in human

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**Figure 3.** A, The tail moments from a comet assay performed 1 minute after 500 rad (5 Gy) of radiation in a patient with cervical lymph node metastases are represented in histogram form (left), with the tail moments on the x-axis and the percentage of the comets on the y-axis. Histograms shifted to the right occur when the tumors are well oxygenated, allowing greater DNA damage during irradiation. The graph on the right depicts the raw data, including the comet tail moments plotted against the DNA contents. B, Two minutes after irradiation, the histogram has shifted to the left, with a lowering of the median tail moment. C, Finally, at 3 minutes, a further shift to the left is apparent, reflecting incremental DNA repair.
found (for a correlation between the 2 variables, but none was influencing DNA repair rate, we explored our data to determine if patient age might be a confounding variable. The comet assay is a promising tool for evaluating radiation sensitivity in individual cells. Our data regarding estimated DNA repair rates early after irradiation are consistent with expectations, and further validate this technique.

CONCLUSIONS

The comet assay is a promising tool for evaluating radiation sensitivity in individual cells. Our data regarding estimated DNA repair rates early after irradiation are consistent with expectations, and further validate this technique.

Accepted for publication November 13, 2001.

This study was supported in part by grant CA 67166 from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

This study was presented in part at the Fifth International Congress on Head and Neck Cancer, San Francisco, Calif, July 29, 2000.

The image analysis for this study was performed using software developed by Ralph Durand, PhD, University of British Columbia, Vancouver.

REFERENCES