The Comet Assay in Clinical Practice

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Acta Oncologica Vol. 38, No. 7, pp. 839–844, 1999

The comet assay is a single-cell gel electrophoresis technique that measures DNA damage in individual cells. Since radiation produces 3–4 times more DNA damage in well-oxygenated cells compared with hypoxic cells, this assay can quantify the fraction of radiation-resistant hypoxic cells found in many solid tumours. This paper summarizes our results with 73 accessible metastatic tumours irradiated with palliative intent. Hypoxic fractions ranged from 0.0 to 0.67 with a mean of 0.15; 62% of these advanced tumours showed a hypoxic fraction > 0.05. Comparisons between two sequential aspirates in 33 tumours gave a slope of 0.92 ($r^2 = 0.88$), suggesting that a single aspirate is generally representative of the tumour. A limitation, however, is that the hypoxic fraction could not be measured in clinical samples given a conventional dose of 2 Gy.

Received 28 July 1998
Accepted 23 February 1999

The comet assay, introduced in 1984 by Ostling & Johanson (1), has become a popular way of detecting a variety of types of DNA damage in individual cells (2–4). The unique feature of this method is the ability to measure heterogeneity in response to drugs and radiation (5). This provides an opportunity to identify resistant cells within the population, and to use DNA damage as a possible predictor of response to treatment. Since so few cells are required for analysis, a single fine-needle aspirate biopsy is generally sufficient. The method is applicable to any cell, making it possible to measure the response of not only tumour, but also normal tissues (e.g., peripheral lymphocytes, bone marrow cells, hair follicle cells, bladder washings, and skin fibroblasts).

Previous applications of the comet assay to clinical samples include measurement of tumour hypoxia (6–8), measurement of tumour growth fraction (9, 10), detection of drug response (11–13), detection of apoptotic cells (11), and analysis of DNA double-strand break rejoining capacity in vitro (14). In the last case it was found that cells from fine-needle aspirates were not capable of rejoining significant numbers of double-strand breaks, presumably because they had been damaged during the process of aspiration. However, sampling cells from patients at some time after exposure allows evaluation of several factors that might contribute to response in vivo (e.g., hypoxia, pH, nutrient status, drug delivery, repair).

Detection of radiation-resistant hypoxic cells is one of the unique applications of the comet assay; the basis for this application is that radiation produces 3–4 times more DNA damage in well-oxygenated cells than in radiobiologically hypoxic cells (6–8). Unlike other techniques, the comet assay is specific for whole nucleated cells and is thus largely unaffected by necrosis, and it measures radiobiologic hypoxia during irradiation. In murine tumours, the proportion of cells that are hypoxic by this assay predicts for tumour cure by radiation (15). However, there is an important limitation in using the comet assay for detection of hypoxic cells. The patient must receive a dose of radiation in excess of 3.5 Gy in order to produce sufficient DNA damage to resolve a hypoxic (radioresistant) population. There are two other potential limitations to the use of the comet assay in the clinic. It is necessary to separate the response of diploid tumour cells from that of circulating white blood cells that contaminate the sample, and the biopsy must be representative of the tumour. In this paper we address these three issues and examine possible solutions.

 ISSN 0284-186X

MATERIAL AND METHODS

Cell samples

Fine-needle aspiration biopsy (FNAB) was carried out in 73 patients with accessible metastatic tumours undergoing palliative radiotherapy for advanced disease. Descriptions of tumour types (6–8) and the method used for aspiration (8) have been published. SCCVII squamous cell carcinoma was grown subcutaneously on the dorsum of C3H/HeJ mice. Tumours were evaluated for hypoxic fraction when they had reached a size of approximately 500 mg. Irradiation was performed with a 250 kV x-ray at a dose rate of 3.3 Gy/min.

Comet assay

FNAB produced a single-cell suspension, so no further manipulation was required before diluting to a concentration of 40000 cells/ml. Single cells (20000 were embedded in 0.75% low gelling temperature agarose containing 2% DMSO and spread on agarose-coated microscope slides. Slides were carefully submersed in an alkaline lysing solution as previously described (8). Following lysis and rinse, slides were electrophoresed at 0.6 volts/cm for 25 min, and then stained for 20 min in 2.5 μg/ml propidium iodide. For each sample, approximately 800 individual cells or 'comets' were observed using a Zeiss epifluorescence microscope and image analysis system (16). DNA damage was quantified as an increase in tail moment, an indicator of damage that is proportional to the number of strand breaks per cell. Tail moment is the product of the percent of DNA (fluorescence) in the tail and the distance between the means of the head and tail fluorescence distributions.

Analysis of hypoxic fraction

Frequency histograms for tail moment were used to calculate hypoxic fraction. An iterative fitting technique, independent of the amount of DNA damage, was used to determine the size and position of the two normal distributions representing the aerobic and hypoxic populations. For some histograms, a third normal distribution was used to describe the population of heavily damaged cells. The displacement between the aerobic and hypoxic peaks was constrained to fall between 1.9 and 3, and a free fit to the data was performed using a least squares approach.

Cell separation using magnetic immunobeads

Two FNAB biopsies were obtained from a patient undergoing palliative radiotherapy for metastatic adenocarcinoma of the breast. The biopsies were taken from a subcutaneous nodule immediately following exposure of the tumour to 5 Gy x-rays. The samples were divided into two parts for treatment with or without magnetic bead separation prior to analysis of DNA damage using the comet assay.

Dynabeads directly conjugated with CD45 (cells bearing leucocyte common antigen) or CD15 (myeloid cells) were purchased from Dynal Inc., Lake Success, NY. The beads were washed and diluted according to the manufacturer's instructions prior to incubation with tumour cells at a ratio of 5 : 1 in sterile phosphate buffered saline containing 1% bovine serum albumin. Samples were incubated in a cold room for 45 min using a rotating mixer. A magnet (Dynal MPC-1) was then used to pull the beads along with bound WBCs (white blood cells) to the side of the test tube. After 2–3 min, the enriched tumour cell suspension was decanted into a tube. Tumour cells were diluted to a density of 2×10^4 cells/ml for the comet assay.

RESULTS

Hypoxic cells were identified in the majority of FNABs from advanced metastatic tumours. The range of responses from 73 tumours measured for hypoxic fraction using the alkaline comet assay over a 5-year period is shown in Fig. 1; 38% of tumours showed a hypoxic fraction ≤ 0.05 of which 29% showed no evidence of hypoxic cells. Three representative examples of histograms showing DNA damage are presented in Fig. 2. In sample (a), most of the cells from a lung metastasis are hypoxic. In sample (c), cells from a melanoma are all well oxygenated. In sample (b), a small hypoxic population is present along with a large percentage of heavily damaged cells. These cells contained more than three times the damage expected from 8 Gy. Comparable populations of heavily damaged cells ($> 20\%$ of the FNAB) occur in about 25% of tumour samples, and can limit ability to resolve the hypoxic cell population (8). The fraction of damaged cells increases with time after irradiation, and, like accumulation of cells in G2 phase, might be useful as a crude indicator of in vivo response (7).

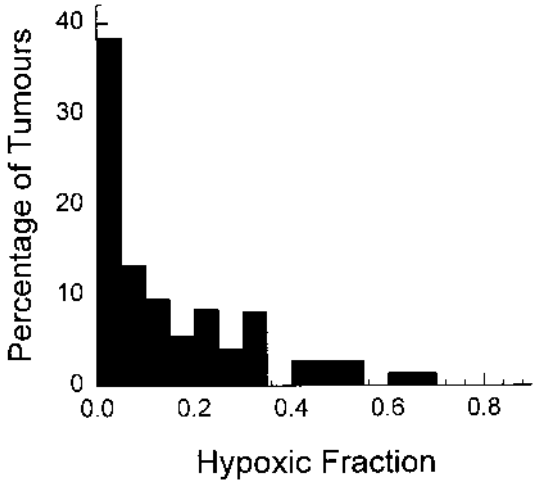

Fig. 1. Distribution of hypoxic fractions measured in human tumours using the alkaline comet assay. Results are shown for 73 tumours measured over 5 years.

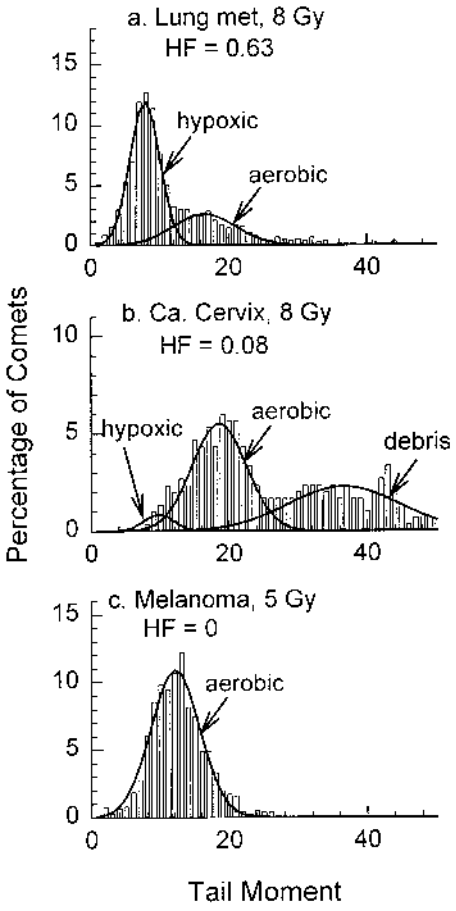

Fig. 2. Appearance of comet histograms and analysis of hypoxic fraction using the alkaline comet assay. Three representative DNA damage histograms are shown for cells obtained from a single fine-needle aspirate biopsy. Hypoxic fraction was calculated by iterative fitting of histograms with 2 or 3 normal distributions representing hypoxic, aerobic and damaged populations (15).

Detection of hypoxic cells requires the exposure of tumours to a dose > 3.5 Gy immediately before aspiration biopsy. While it is possible to detect hypoxic cells in the SCCVII mouse tumour following exposure to 2 Gy, the dose rate must be high, and tumour cells must be recovered rapidly after irradiation (Fig. 3c, d). We have noted a tendency for the hypoxic fraction to be somewhat higher at these lower damage levels, which may indicate a decreased precision for identifying minimally damaged cells as a separate population. We have not been successful in identifying hypoxic cells after 2 Gy exposures in the clinic; typical responses following treatment with 2 Gy can be found in Fig. 3a, b.

Although a single FNAB contains sufficient cells for the comet assay to be performed, the question arises whether one aspirate can be representative of the tumour. In dealing with this question, we compared the hypoxic fraction measured using two aspirates from 33 patients (Fig. 4). With the exception of an obvious outlier (omitted from the

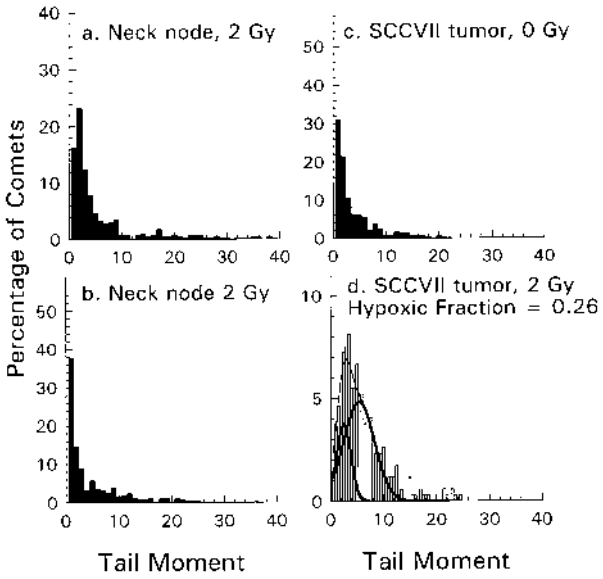

Fig. 3. Measurement of hypoxic cells in tumours following exposure to 2 Gy. Panels a and b show the response of cells from the neck nodes of two patients. Panels c and d show the response of the murine SCCVII tumour to 0 Gy or 2 Gy.

regression analysis), there was a very good correlation between the responses measured in two successive aspirates. Tumours that appeared poorly oxygenated in the first sample (hypoxic fraction > 0.3) were also poorly oxygenated in the second sample. However, for some tumours, there was a considerable range between the two measurements so that it would be difficult to characterize a tumour as aerobic or hypoxic below a hypoxic fraction of 0.3.

Circulating WBCs can represent a significant proportion of the cells obtained by FNAB. Since these cells are not exposed to the full dose of radiation, they typically appear

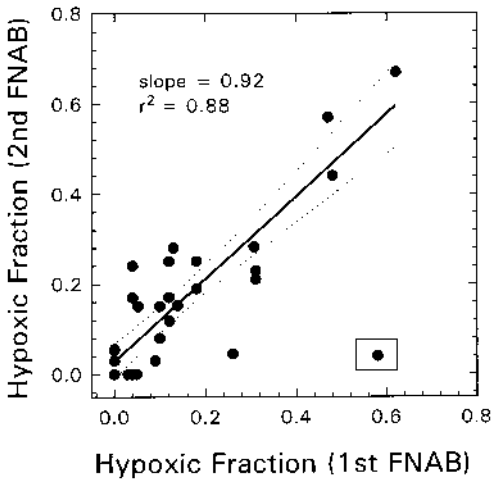

Fig. 4. Comparison of hypoxic fraction measured in two sequential aspirates from the same tumour. The outlier in the box has not been included in the analysis.

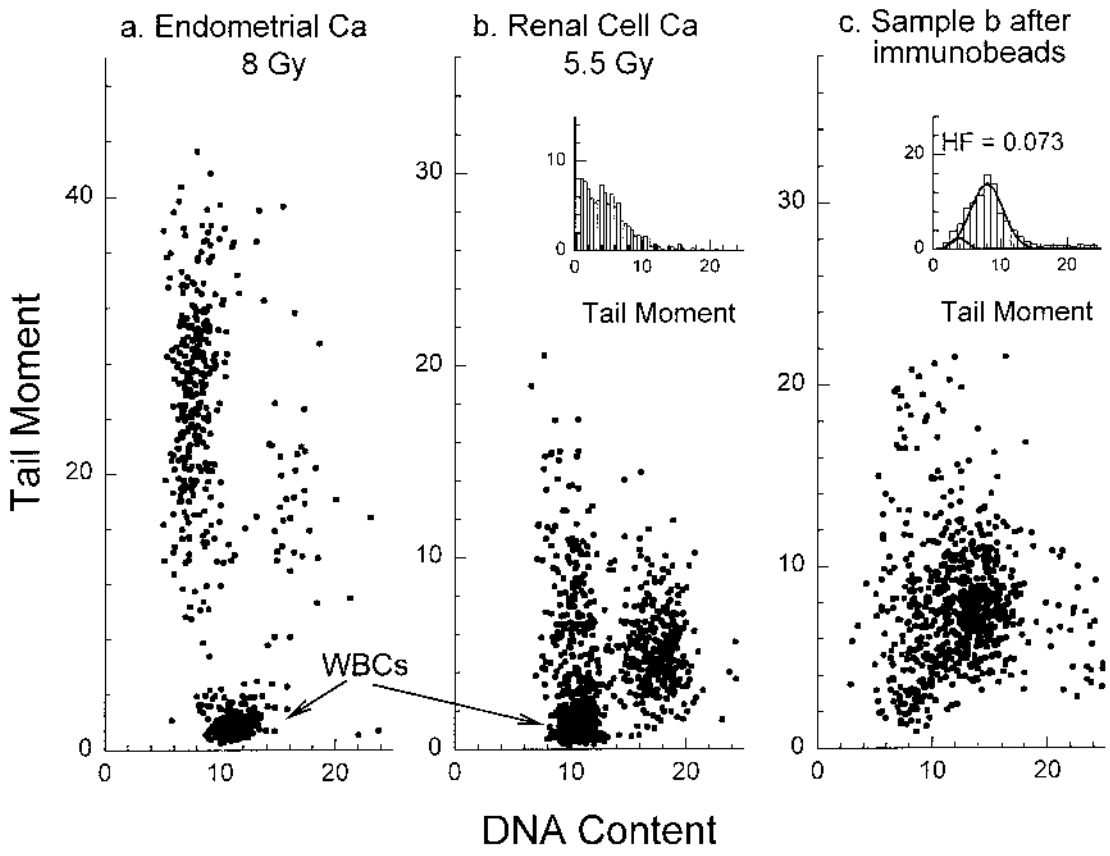

Fig. 5. Use of magnetic immunobeads to enrich aspirates for tumour cells. Fine-needle aspirates were incubated with magnetic immunobeads coated with antibodies against leucocyte cell surface antigens. A magnet was used to separate tumour cells and leucocytes and the alkaline comet assay was performed. Panel a: 8 Gy irradiated tumour sample showing a distinct population of undamaged cells. Panel b: A sample of irradiated cells from a renal cell carcinoma metastasis analysed using the comet assay without magnetic bead separation. Panel c: The same renal carcinoma sample, but shown after magnetic immunobeads were used to remove leukocytes. Inserts in panels b and c show the frequency of comets with specific tail moments.

as an undamaged population of cells with diploid DNA content. If sufficiently distinct from the irradiated population (Fig. 5a), or if the tumour cells are aneuploid, WBCs constitute no problem for analysis of hypoxic fraction. However, when smaller radiation doses are used, the leucocytes can be difficult to distinguish from hypoxic diploid tumour cells. A simple method to overcome this problem (practical for a limited sample size) is to use magnetic immunobeads coated with antibodies against leucocyte cell surface antigens (17). After incubation, the beads can be removed and discarded, providing a rapid way to enrich for tumour cells in the aspirate. The results from a patient with a renal cell carcinoma, metastatic to the upper arm, who received 5.5 Gy irradiation in a single fraction are presented in Fig. 5b and c. In Fig. 5b, a large proportion of these cells have low levels of DNA damage and could be leucocytes. It was not possible to determine a hypoxic fraction from the tail moment histogram in Fig. 5b. After incubation with magnetic immunobeads, the proportion of cells with low amounts of damage was decreased. More importantly, the proportion of cells with higher DNA content was significantly increased, consistent with depletion of leucocytes and enrichment of the tumour cell population. Interestingly, the hypoxic fraction calculated for those cells in Fig. 5b with a DNA content > diploid (an arbitrary value of 13 in panel b) was 0.092, which is similar to the results shown in Fig. 5c after enrichment. This confirms that the enrichment process increased the percentage of tumour cells without altering the proportion that were hypoxic.

DISCUSSION

The popularity of the comet assay in clinical analysis and biomonitoring owes much to the relative simplicity and rapidity of the method, combined with the important practical factor that few cells are required for analysis. However, there is always a concern that a fine-needle aspirate will not be representative of the tumour. In fact, our results show a good correlation between two sequential aspirates. Therefore, despite the small volume of the sample obtained by this method, the procedure used to obtain the sample (rapid oscillating movement of a 25-

gauge needle throughout the tumour mass) is usually adequate for obtaining a representative sample. In only one of the 33 pairs of aspirates was there a very large discrepancy in the two samples. Interestingly, the DNA content of the cells in these two samples was also significantly different. However, there was a greater than 5% variation between many of the pairs, reflecting not only sampling differences but also errors associated with curve fitting required to obtain a hypoxic fraction.

Approximately 62% of 73 advanced tumours showed evidence of hypoxic cells (hypoxic fraction > 0.05) when analysed by the alkaline comet assay. Recently, however, we compared the comet assay with the oxygen electrode measurements made in the same human tumour. For tumours defined as hypoxic based on a median oxygen tension < 10 mmHg, the percentage of hypoxic cells measured by the comet assay was $> 20\%$ (18). The percentage of tumours that fall into this category in Fig. 1 is only 31%. This can be compared with approximately 40% of a variety of tumours from patients given iodoazomycin arabinoside prior to SPECT analysis (19), 97% of tumours analysed by ^{18}F-PET (20), or 50% of advanced cervix carcinoma analysed by Eppendorf oxygen electrode (21). The wide range in comet hypoxic fraction (0–0.67) is consistent with results using these other methods (20).

Can we improve the sensitivity of the comet assay so that a clinically relevant dose of 2 Gy can be used to detect hypoxic cells? We have attempted to improve the sensitivity by application of purified enzymes that recognize DNA base damage. Results indicate that this procedure increases sensitivity about twofold, but unfortunately it also reduces the ability to resolve a hypoxic fraction because the oxygen enhancement ratio for DNA base damage is only 1.0 (22). Even if the comet assay for hypoxia requires doses in excess of 3.5 Gy, many questions can be addressed with accessible metastatic tumours. Which treatments promote tumour reoxygenation? Which drugs eliminate hypoxic cells? Can we use the comet assay to help calibrate or interpret results using hypoxia markers, oxygen electrodes and non-invasive methods for hypoxia detection?

The question of maximizing the 'signal to noise' ratio in an FNAB has been addressed using magnetic immunobeads to enrich aspirates for tumour cells. Preliminary results shown here for one patient and confirmed in two others will require validation using known mixtures of leucocytes and tumour cells. Routine application of this method could significantly improve the reliability of clinical data obtained using the comet assay.

In this paper we emphasize the application of the comet assay to the question of tumour hypoxia since most of our clinical data have been accumulated for that purpose. However, many areas of clinical investigation could benefit from application of this method. DNA damage can be used to detect drug-resistant cells within tumours (23, 24) or to measure the effects of agents that modify drug resistance (25). Of course, this approach will be limited to those drugs that produce DNA damage that can be measured by the comet assay. For those drugs where DNA damage is predictive of cell killing, tumour response might be compared to normal tissues to provide an estimate of therapeutic ratio (26). DNA repair capacity and apoptosis can be measured in peripheral blood cells (11, 27, 28) and perhaps used as an index of the drug or radiation sensitivity of the patient. The comet assay can also be used as a basis for prenatal diagnosis of the UV repair deficient disorder, *Xeroderma pigmentosum*, by using fetal cells from amniotic fluid (29). Even this brief list of clinical applications suggests that this versatile method will continue to find new applications in clinical practice.

ACKNOWLEDGEMENTS

This work was supported by NIH grant CA 37879, and by the National Cancer Institute of Canada with funds provided by the Canadian Cancer Society.

REFERENCES

1. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 1984; 123: 291–8.
2. Fairbairn DW, Olive PL, O'Neill KL. The comet assay: a comprehensive review. Mutat Res 1995; 339: 37–59.
3. McKelvey-Martin VM, Green MHL, Schmezer P, Pool-Zobel BL, DeMeo MP, Collins A. The single cell gel electrophoresis assay (Comet assay): a European review Mutat Res 1993; 288: 47–63.
4. Schmezer P. The Comet Assay. Special issue of Mutat Res 1997; vol 375, no 2. Amsterdam: Elsevier.
5. Olive PL, Johnston PJ, Banath JP, Durand RE. The comet assay: a new method to examine heterogeneity associated with solid tumours. Nature Med 1998; 4: 103–5.
6. Olive PL, Durand RE, LeRiche J, Olivotto I, Jackson SM. Gel electrophoresis of individual cells to quantify hypoxic fraction in human breast cancers. Cancer Res 1993; 53: 733–6.
7. Olive PL, Trotter T, Banath JP, Jackson SM, LeRiche J. Heterogeneity in human tumour hypoxic fraction measured using the comet assay. Br J Cancer 1996; 74: 191S–5S.
8. McLaren DB, Pickles T, Thomson T, Olive PL. Impact of nicotinamide on human tumour hypoxic fraction measured using the comet assay. Radiother & Oncol 1997; 45: 175–82.
9. Olive PL, Banath JP. Growth fraction measured using the comet assay. Cell Prolif 1992; 25: 447–57.
10. Olive PL, LeRiche J, Jackson SM. Growth fraction of human tumours: assays and complications. Semin Radiat Oncol 1993; 7: 90–8.
11. Tice RR, Strauss GH, Peters WP. High-dose combination alkylating agents with autologous bone-marrow support in patients with breast cancer: preliminary assessment of DNA damage in individual peripheral blood lymphocytes using the single cell gel electrophoresis assay. Mutat Res 1992; 271: 101–3.
12. Vaghef H, Nygren P, Edling C, Bergh J, Hellman B. Alkaline single-cell gel electrophoresis and human biomonitoring for genotoxicity: a pilot study on breast cancer patients undergoing chemotherapy using cyclophosphamide. Mutat Res 1997; 395: 127–38.

13. Hartmann A, Herkomer K, Gluck M, Speit G. DNA damage effect of cyclophosphamide on human blood cells in vivo and in vitro studied with the single-cell gel test (comet assay). Environ & Molec Mutagen 1995; 25: 180–7.
14. Olive PL, Jackson SM, Durand RE. Predicting tumour response to radiotherapy using the comet assay. In: Paliwal BR, Kinsella TJ, Herbert D, Fowler J, eds. Proceedings of the 4th International Conference on Time, Dose and Fractionation in Radiation Oncology. Woodbury (NY): The American Institute of Physics, 1993: 65–76.
15. Olive PL, Horsman M, Grau C, Overgaard J. Detection of hypoxic cells in a C3H mouse mammary carcinoma using the comet assay. Br J Cancer 1997; 76: 694–9.
16. Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumour and normal cells measured using the 'comet' assay. Radiat Res 1990; 122: 69–72.
17. Strauss GHS, Peters WP, Everson RB. Measuring DNA damage in individual cells of heterogeneous mixtures: a novel application of an immunological typing technique. Mutat Res 1994; 304: 211–6.
18. Aquino-Parsons C, Luo C, Vikse CM, Olive PL. Comparison between the comet assay and the oxygen microelectrode for measurement of tumour hypoxia. Radiother & Oncol, in press.
19. Utrasun RC, Parliament MB, McEwan AJ, et al. Measurement of hypoxia in human tumours by non-invasive SPECT imaging of iodoazomycin arabinoside. Br J Cancer 1996; 74: S209–12.
20. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996; 56: 4509–15.
21. Rasey JS, Koh W, Evans ML, et al. Quantifying regional hypoxia in human tumors with positron emission tomography of 18F-fluoromisonidazole: A pretherapy study of 37 patients. Int J Radiat Oncol Biol Phys 1996; 36: 417–28.
22. Banath JP, Wallace SS, Thompson J, Olive, PL. Base damage detected using the alkaline comet assay: oxygen enhancement ratio and influence of single-strand breaks. Radiat Res, in press.
23. Olive PL, Banath JP. Multicell spheroid response to drugs predicted with the comet assay. Cancer Res 1997; 57: 5528–33.
24. Olive PL, Vikse CM, Banath JP. Use of the comet assay to identify cells sensitive to tirapazamine in multicell spheroids and tumours in mice. Cancer Res 1996; 56: 4460–3.
25. Huang P, Olive PL, Durand RE. Use of the comet assay for assessment of drug resistance and its modulation in vivo. Br J Cancer 1998; 77: 412–6.
26. Olive PL. Use of the comet assay to detect hypoxic cells in murine tumours and normal tissues exposed to bioreductive drugs. Acta Oncol 1995; 34: 301–5.
27. McCurdy D, Tail LQ, Frias S, Wang Z. Delayed repair of DNA damage by ionizing radiation in cells from patients with juvenile systemic lupus erythematosus and rheumatoid arthritis. Radiat Res 1997; 147: 48–54.
28. Banath JP, Fushiki M, Olive PL. Rejoining of DNA single-and double-strand breaks in human white blood cells exposed to ionizing radiation. Int J Radiat Oncol 1998; 73: 649–60.
29. Alapete C, Benoit A, Moustacchi E, Sarasin A. The comet assay as a repair test for prenatal diagnosis of Xeroderma pigmentosum and trichothiodystrophy. J Invest Dermatol 1997; 108: 154–9.