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Original Paper

Determination of Radiation-induced Damage in Lymphocytes using the Micronucleus and Microgel Electrophoresis 'Comet' Assays

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DNA damage assays may be useful as rapid predictors of normal tissue radiosensitivity in clinical samples. We measured *in vitro* radiation-induced (2 Gy) damage to lymphocytes from cancer patients and normal healthy donors using both the micronucleus and microgel electrophoresis (Comet) assays simultaneously. For damage assessment, there was a good correlation ($P < 0.001$) between the mean comet lengths and the fraction of cells with comets. There was no correlation with initial damage, determined as the proportion of cells within a sample that formed comets, in comparison with the mean frequency of micronuclei per binucleate cell. However, there appeared to be an association between the determination of repair proficiency in the Comet assay and the mean frequency of micronuclei per binucleate cell in lymphocytes from cancer patients.

Key words: lymphocytes, radiosensitivity, normal tissue tolerance, DNA damage, DNA repair, micronucleus, electrophoresis, agar gel

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INTRODUCTION

PREDICTORS OF intrinsic radiosensitivity [1] are of clinical relevance since individual patients vary in their response to radiation treatment [2]. The extent of radiotherapy is governed by considerations of both the response of tumour cells and the tolerance of the surrounding normal tissues—thus, particularly radiosensitive individuals determine the maximum tumour dose. If radiosensitive patients could be identified, the remaining patients could possibly receive an increased dose with associated therapeutic gain [3]. Rapid predictive assays that can detect extreme responders [4] would have a valuable clinical role and contribute to the aim of individualised treatments of patients based on biological considerations.

We have previously used a nuclear lysate sedimentation technique [5] to assess the repair proficiency (at 1 h) of lymphocytes, following *in vitro* irradiation (2 Gy) from carcinoma of the cervix patients about to or having undergone radiotherapy. At 2 years post radiotherapy, a high proportion (44%) of patients with late bowel damage (attributable to radiation) had lymphocytes with poor repair proficiency compared to none in the group described as 'well and complication-free'. Only 7% of healthy donors and pre-radiotherapy patients had lymphocytes with poor repair proficiency. We have also shown that the cytokinesis-block micronucleus (CBMN) assay, when used to measure radiosensitivity in tumour cells, does not correlate with cellular survival [6]. Recently, however, it has been suggested that the micronucleus assay can be used for normal cells in order to determine the intrinsic radiosensitivity of individuals [7].

As part of an ongoing study of predictive assays for normal tissue radiosensitivity, we have examined the use of a modified (CBMN) assay [7, 8] and a modified Comet assay [9, 10]. Here, using lymphocytes, we attempted to determine whether the 'non-repaired' DNA damage measured as micronuclei is associated with DNA damage or repair proficiency measured in the Comet assay.

MATERIALS AND METHODS

The responses of lymphocytes from patients with squamous cell carcinoma of the head and neck (8), uterine cervix (2) or from healthy donors (5) were studied.

Lymphocytes were separated (Histopaque-1077 gradient, Sigma Chemical Co., U.K.) from heparinised whole blood (30 ml), washed and re-suspended in RPMI 1640 (Life Sciences International, U.K.) supplemented with 10% fetal calf serum (Globe Pharmaceuticals, U.K.) and antibiotics (200 U/ml penicillin and 100 μg/ml streptomycin, Sigma). Samples were divided for simultaneous analysis in the CBMN and Comet assays.

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Cells were irradiated on a ^{137}Caesium source (gamma-irradiation, 0.66 MeV) at a dose rate of 0.79 Gy/min, either in sterile plastic tubes containing 0.8 ml of cell suspension for the micronucleus assay (1–2.5 Gy) or embedded in a thin layer of 0.5% agarose in medium (75 μl) mounted on a microscope slide for the Comet assay (2 Gy). The embedded cells were sandwiched between 150 μl agarose layers formed using a 22 × 50 mm coverslip.

In the micronucleus assay, Cytochalasin-B (Sigma) was added 24 h after irradiation and stimulation with Phytohemagglutinin-C (IBF-Biotechnics, France). After 72 h total culture time, duplicate slides were stained with Jenner-Giemsa (BDH Laboratory Supplies, U.K.). Cells (1000 in total) were scored using the criteria described by Almássy and associates [11] except that micronuclei were also scored if close to a nucleus that was otherwise round and without blebbing. DNA damage was represented as the mean frequency of micronuclei per binucleate cell (mean MN/BNC).

In the Comet assay, cells were either lysed immediately after irradiation (to determine initial damage) or after incubation in a humidified CO_2 incubator at 37°C for 1 h (repair). Following lysis (5 min in 2.5 M NaCl, 1% sodium sarcosinate, pH 10) of the agarose-embedded cells, electrophoresis (30 min, 0.86 V/cm, 300 mA) was conducted under alkaline conditions (300 mM NaOH, 1 mM Na–EDTA, pH 13) using a BioRad submerged gel electrophoresis system (15 × 33 cm). After staining with ethidium bromide, DNA damage was measured either as mean comet length (centre of head to tail end) or as the fraction of comets with tails longer than 30 μm. Between 50 and 100 cells/comets were assessed from photographic images obtained using a Leica Aristoplan fluorescence microscope (×16 objective). The repair proficiency was calculated as (damage − repair)/(damage − non irradiated), that is, the ratio of extent of comet restoration to the extent of radiation-induced damage.

RESULTS

The response to *in vitro* irradiation of lymphocytes from 15 donors (5 healthy donors, 10 patients) was measured using both the micronucleus and comet microgel electrophoresis assays. The range of mean MN/BNC observed in two representative donors is shown in Figure 1. Figure 2 compares the distribution of comet lengths following 2 Gy irradiation (damage) and incubation (repair) from the same 2 donors. In the Comet assay, with the exception of 1 patient, the non-irradiated samples consisted predominately of cells which did not form tails during electrophoresis. The 1 patient had 86% of cells forming comet tails whilst the range for the remaining 14 patients and healthy donors was between 7.5 and 43% (mean 26% ± 12 S.D.). For all 15 donors, the range of cells forming tails following *in vitro* irradiation varied between 52 and 90% (mean 75% ± 12.7 S.D.). In the Comet assay, damage was expressed as either the mean of the individual comet lengths or the proportion of cells which formed comet tails. A regression line, based on the repair proficiency value, showed a good ($y = 0.21720 + 0.64792x$, $r = 0.897$, $P < 0.001$) correlation between these two parameters. The errors (due to the bimodal distribution of comet tail lengths) associated with the use of the mean comet length led us to consider the estimate of repair based on the proportion of cells that formed comet tails of greater than 30 μm. The cut-off point of 30 μm was arbitrarily chosen based on the histogram data of comet lenghts such as those illustrated in Figure 2.

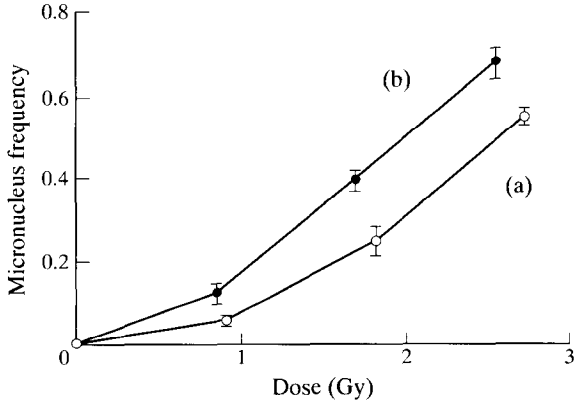

Figure 1. The dose response of human lymphocytes from 2 patient donors measured using the micronucleus assay. The examples were selected on the basis of extreme responders within the groups' donor (a) low mean MN/BNC and donor (b) high mean MN/BNC over the range 1–2.5 Gy. Error bars indicate the standard error.

A comparison of Figures 1 and 2 shows that donor (a) with the lower mean MN/BNC demonstrated a good repair proficiency (0.854) whereas donor (b) with the higher mean MN/BNC demonstrated no repair in the Comet assay. There was no correlation between the mean MN/BNC and the Comet assay assessment of initial or residual damage. However, comparison of the micronucleus frequency with comet repair proficiency suggested an inverse relationship (Figure 3) with greater residual damage (MN/BNC) associated with a poor repair proficiency in 12 of 15 samples. An association appears to exist between the samples taken from the patients. In Figure 3 a simple regression line of the patient data ($r = 0.842$) is shown to indicate the trend that may exist in the patient data. Three of five samples from 'normal' healthy donors showed a good repair proficiency but were associated with a greater micronucleus frequency. There was a wider range of values for repair proficiency (0–1) than for micronucleus frequency (0.251–0.401).

DISCUSSION

The micronucleus assay detects DNA damage as whole or fragmented chromosomes that have not been repaired at the time of cell division. In the Comet assay, detection of DNA damage can be described in a number of ways including tail:head ratio [12] and tail moment [13], but tail length is regarded as reasonably valid over the low dose range where essentially the quality and appearance of the tails are similar [14–16]. In Figure 2, there are two distinct populations consisting of (i) 'cells' with no or short tails and (ii) a normal distribution of 'cells' with comet tails. Given the distribution of comet lengths, we considered that it was not a rigorous approach to use the mean of the comet lengths when describing the damage within a sample. However, the proportion of cells that formed DNA tails correlated well with the mean comet length. Given the large errors associated with the distribution of comet lengths, we used the proportion of cells that formed DNA tails to obtain an estimate of repair proficiency. In the Comet assay, the study of repair kinetics suggests that most damage is repaired within the first 15 min of incubation, with a second slower repair completed after 120 min [10, 12]. Previously, using a nuclear lysate sedimentation technique, we found that a 1 h post-irradiation incubation at 37°C resulted in good repair of most samples of lymphocytes [5]. Thus, in our current study, most of the damage could be expected to be repaired during the 1 h post-irradiation incubation—the term 'repair' indicating an increase in the

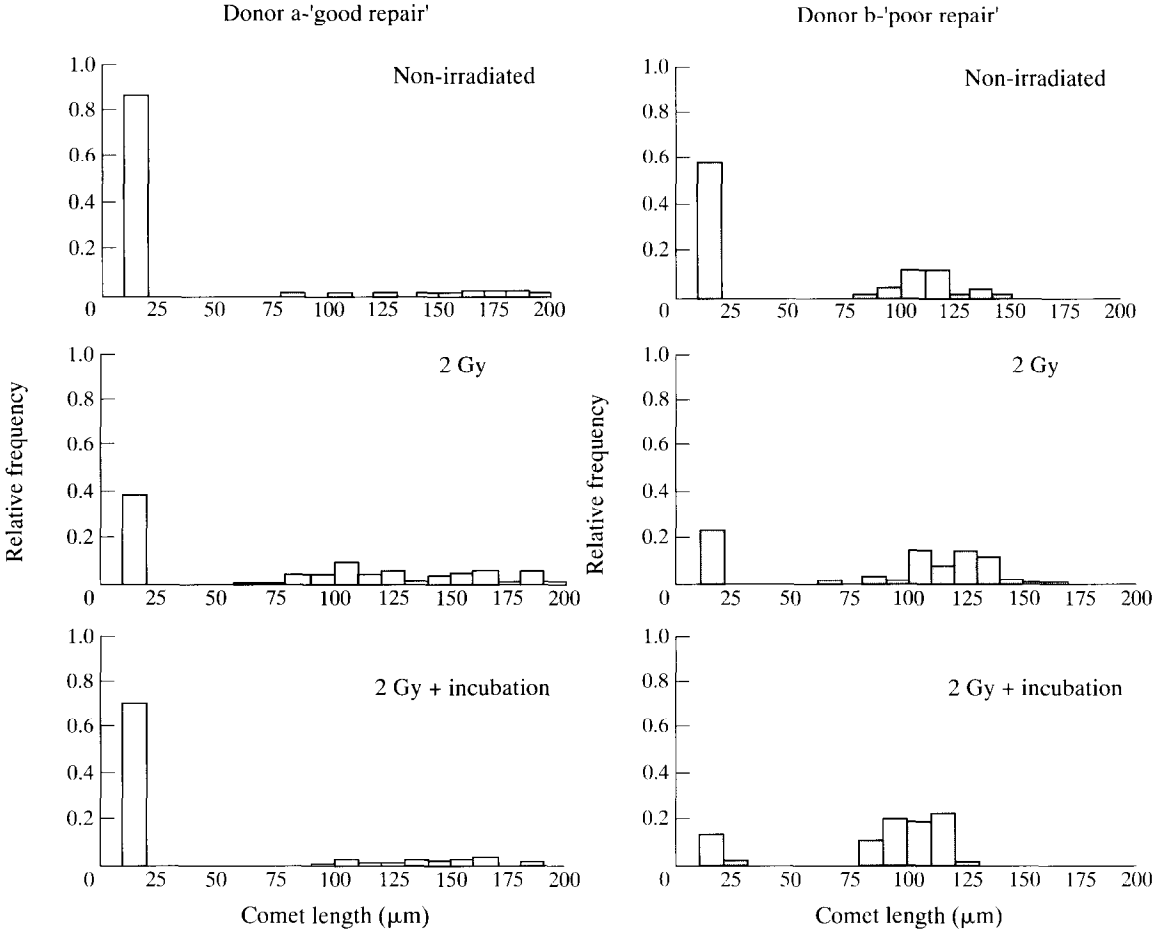

Figure 2. Comparison of distribution of comet tail lengths in non-irradiated, 2 Gy irradiated (damage) and 2 Gy irradiated followed by 1 h incubation at 37°C (repair) in the 2 donors presented in Figure 1.

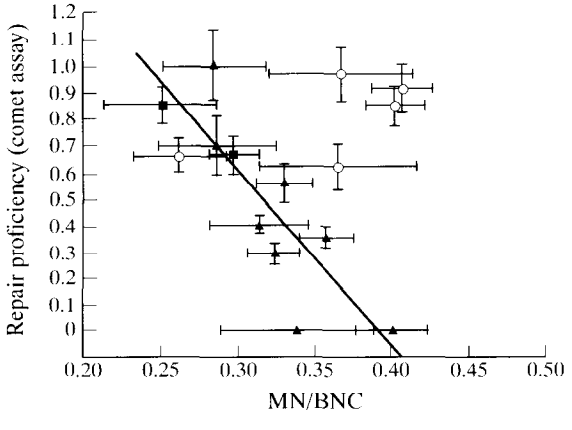

Figure 3. Comparison between the micronucleus frequency and coment repair proficiency for 5 healthy donors (○) and 10 patients with squamous carcinoma of either the head and neck (▲) or uterine cervix (■). A simple regression line of the patient group ($y = 2.6125 - 6.6869x$, $r = 0.897$) is shown to indicate the trend in the association within the patient group. Error bars indicate the standard error.

proportion of cells which did not form comet tails upon electrophoresis.

Whilst repair fidelity [17] and not proficiency may be more relevant to cell survival, comparison of these two short term assays (Figure 3) suggests that there may be an association between repair proficiency (Comet assay) and micronucleus frequency. Figure 1 shows dose responses, over the 1–2.5 Gy, for lymphocytes of 2 patients measured using the micronucleus assay. Currently, these represent the extreme responders within our small sample of patients and suggest that a relatively small range (of about 1.6 times) exists in the micronucleus response. In contrast, in Figure 3 there appears to be no similar association for the healthy donor group. The use of the lymphocyte to reflect variability in normal tissue at risk during radiotherapy may be limited. However, Floyd and Cassoni [7] suggested that the literature reports of clonogenic capacity showed human cord blood lymphocytes to have a greater radiosensitivity than normal lymphocytes and that a similar conclusion could be drawn using the micronucleus assay. Thus, they argued that the micronucleus assay could be used to determine intrinsic radiosensitivity and so identify radiosensitive individuals. The validity of their claim remains to be examined.

In conclusion, the possible relationship between the micronucleus assay and other assays of DNA damage such as the Comet assay needs to be better understood. We are currently continuing our study to complete an evaluation of the suitability of these

two assays to determine radiosensitivity and predict individuals at risk.

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