Modification of the alkaline Comet assay to allow simultaneous evaluation of mitomycin C-induced DNA cross-link damage and repair of specific DNA sequences in RT4 cells

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Abstract

The alkaline Comet assay is a simple, sensitive method for measuring the extent of DNA strand breaks in individual cells. Several modifications to the original assay have been developed to increase its applications. One such modification allows the measurement of DNA cross-links by assessing the relative reduction in DNA migration induced by a strand-breaking agent. Another modification includes the application of fluorescent in situ hybridisation (FISH) to investigate the localisation of specific gene domains within a cell. Although several studies have used these approaches separately, no report to date has combined these two versions of the Comet assay. The current study describes the modification of the Comet assay, to allow both measurement of mitomycin C (MMC)-induced cross-links and the subsequent application of FISH to study repair in the TP53 gene region. RT4 human bladder cancer cells were treated with 0, 5, 50 and 200 μg/ml MMC to study dose response, whilst for cross-link repair studies, they were treated with 50 μg/ml MMC and allowed to repair for up to 24 h. A clear dose response to MMC was displayed, demonstrable by a marked reduction in DNA migration, whilst repair studies showed that MMC-induced cross-links take at least 24 h to repair fully in RT4 cells. For Comet–FISH experiments, the number and location of TP53 hybridisation spots was also recorded for each cell. In dose response experiments, the number of spots per cell, and per Comet tail, decreased as MMC dose increased. In repair experiments, the number of spots, particularly in the Comet tail, increased as repair time increased. Furthermore, our results suggest that repair of the TP53 gene region is most rapid within the first 4 h following MMC treatment. We conclude that the novel experimental protocol presented here has considerable potential in evaluating DNA damage and sequence-related repair responses to cross-linking agents.

1. Introduction

The alkaline single cell gel electrophoresis (Comet) assay is a simple, sensitive and relatively inexpensive way to measure the extent of DNA strand breakage in individual cells [1,2]. However, it does not readily detect DNA cross-links (i.e. DNA–protein cross-links, DNA inter- and intrastrand cross-links) under the normal conditions of the assay [3]. To address this limitation, modifications to the standard alkaline Comet protocol have been proposed, in which cells are treated with a combination of cross-linking agents and agents
which induce DNA strand breaks. Hence, the extent of DNA cross-linking by a particular agent can be indirectly measured by analysing the relative reduction of DNA migration induced by a strand-breaking agent [4–6].

A further modification to the Comet assay can be introduced by combining it with fluorescent in situ hybridisation (FISH) [7,8]. The Comet–FISH assay employs the use of fluorescence-labelled DNA probes which hybridise to a specific gene sequence. Thus, the localisation of a particular gene within a Comet can be established, offering the opportunity to follow gene-specific repair in response to DNA damage. Previous studies in our laboratory have demonstrated that the Comet–FISH assay can be used to examine DNA damage and repair relating to the $TP53$ region in bladder cancer cells following $\gamma$-irradiation [8,9].

Mitomycin C (MMC) is an alkylating agent that mainly induces DNA–DNA interstrand cross-links [2]. MMC is regarded as an important chemotherapeutic agent and is widely used in the treatment of superficial bladder cancer [10]. Previous experiments have used the alkaline Comet assay to measure the extent of MMC cross-linking in V79 Chinese hamster cells but reported limited success [5,6]. Furthermore, the methods employed in these studies are not suitable for the additional application of FISH, as cellular responses induced by the strand-breaking agents used in these studies may interfere with those induced by the MMC, rendering any information about overall repair and gene related repair ambiguous.

In the current study, by using post-lysis $\gamma$-radiation as the strand-breaking strategy, we have modified the alkaline Comet assay protocol to allow both the assessment of MMC cross-links present in damaged and repairing RT4 bladder cancer cells, and the subsequent meaningful application of FISH to study repair specifically relating to an actively transcribed gene.

2. Materials and methods

2.1. Cell-lines and cell culture

RT4 cells were obtained from the American Tissue Culture Collection (Rockville, Maryland, USA). They were cultured in McCoy’s 5A medium, supplemented with 10% foetal bovine serum (FBS) and containing 1% penicillin–streptomycin. The RT4 cell-line is derived from a superficial bladder cancer and is wild type for $TP53$ [11]. The supplier’s datasheet lists RT4 as hyperdiploid and hypotetraploid to hypertetraploid. We have verified this in our own laboratory [9].

2.2. Treatment of cells with MMC

Cells were harvested, centrifuged at 1200 rpm for 3 min and washed twice in 10 ml Ca$^{2+}$- and Mg$^{2+}$-free phosphate buffered saline (PBS). One millilitre cell suspensions (2 $\times$ $10^5$ cells/ml in PBS) were treated with 0, 5, 50 and 200 g/ml MMC (Sigma; CAS Registry number 57-07-7) made up in PBS in separate eppendorfs. The cells were placed at 37°C for 1 h with occasional agitation. After MMC treatment, the eppendorfs were centrifuged at 1200 rpm for 3 min and each cell pellet washed twice in 1 ml PBS and processed for the alkaline Comet assay. For repair studies, cells were resuspended in growth medium (RPMI supplemented with 20% FBS) and allowed to repair for up to 24 h at 37°C with occasional agitation. Cells were then washed twice with PBS as described above. Cell viability was assessed using Trypan Blue both before and immediately after MMC treatment and was found to be consistently >99%.

2.3. The alkaline Comet assay

The alkaline Comet assay was performed following standard protocol [8] with a few modifications. Experimental conditions were strictly maintained between separate experiments to ensure results would be comparable. Dakin fully frosted microscope slides were each covered with 100 $\mu$m of 0.6% normal melting point agarose (prepared in PBS) at 50°C. A 22 mm $\times$ 22 mm coverslip was placed on top and the slide was kept on ice until the agarose had solidified. Each pellet of cells (prepared as described above) was resuspended in 80 $\mu$l of low melting point agarose (LMA) at 37°C. After gently removing the coverslip, the cell suspension was quickly pipetted onto the first agarose layer, the coverslip replaced on top and the slide left on ice to solidify the agarose.

After removing the coverslips, slides were quickly immersed in freshly prepared, cold lysis solution
(2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 added just before use) for 1 h at 4°C. After lysis, the slides were irradiated to induce strand breaks with 5 Gy H₂O₂-radiation at a dose rate of 2 cGy/s using a Cs₁³⁷ source. In each experiment, control slides were prepared with cells which were not treated with MMC and subsequently received either no irradiation or 5 Gy irradiation only. After lysis and irradiation, the slides were drained and placed in a horizontal gel electrophoresis tank side by side. The tank was filled with fresh, cold electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH > 13) to a level approximately 0.25 cm above the slides. The slides were left in the alkaline buffer for 30 min to allow DNA unwinding to occur. Electrophoresis was then conducted at 25 V (0.66 V/cm) and 300 mA for 30 min. Slides were drained, placed on a tray and flooded slowly with three changes of neutralisation buffer (0.4 M Tris, pH 7.5) for 5 min each, to remove alkali and detergents. Slides were drained and stained with 2 g/ml ethidium bromide and left in a humidified chamber at 4°C prior to analysis. All slides were prepared under yellow light and duplicate slides for each treatment were prepared.

To prepare slides for FISH, they were placed in cold 100% ethanol for 30 min following the three washes with neutralisation buffer. They were drained and placed in 0.5 M NaOH for 25 min at room temperature to fully denature the DNA. They were subsequently dehydrated in an ascending series of ethanol solutions (70, 85, 100%; 5 min each) and left to air dry overnight on dry tissue paper in the dark.

2.4. Fluorescent in situ hybridisation (FISH)

FISH was performed on prepared Comet slides according to the manufacturer’s instructions using a locus specific identifier (LSI) spectrum-orange-labelled randomly sheared DNA probe spanning a region of 145 kb containing the TP53 gene (Vysis, Surrey, UK). For each individual slide, a probe mixture, containing 1 μl probe solution, 7 μl LSI hybridisation buffer (Vysis) and 2 μl sterile water, was heated at 73°C for 5 min and quickly added to the slide. A 22 mm × 22 mm coverslip was placed on top and the edges sealed with cow gum rubber cement. Hybridisation took place at 37°C for 16 h in a dark, humidified chamber.

Following hybridisation, the cow gum seals were carefully removed and slides were placed in a solution of 50% formamide and 2 × SSC for 10 min at 45°C. On placing in the solution, the slides were gently agitated to detach the coverslips and this wash was repeated twice, followed by 1 × 10 min wash in 2 × SSC at 45°C and 1 × 5 min wash in 2 × SSC containing 0.1% Igepal. Slides were drained and left to air dry for 10 min before being counterstained with 15 μl 125 ng/ml 4′-6-diamidino-2-phenylindole (DAPI) in antifade mounting solution (Vysis). Slides were analysed immediately to ensure that quenching of DAPI or probe fluorescence did not have time to occur. All reagents were purchased from Sigma, Poole, UK unless otherwise indicated.

2.5. Comet and Comet–FISH analysis

Comet analysis was performed using an epifluorescence microscope (Nikon Eclipse E400) fitted with a Nikon 60× fluor lens (Plan Apo 60:1 N.A. 1.4) and equipped with a Hamamatsu Orca digital CCD camera and a filter set (Chroma HiQ) tuned for ethidium bromide (excitation 510 nm, emission 595 nm). All Comet analyses was performed using the Komet 4.0 digital imaging system (Kinetic Imaging Ltd.), which measures a wide range of densitometric and geometric parameters for each Comet. The primary measurement used in this study was the % tail DNA. Fifty cells were analysed from each slide and measurements from duplicate slides were averaged. The mean of three independent experiments was used to generate each data point.

For Comet–FISH analysis the same microscope and camera settings were used as stated above. A double bandpass filter set (Chroma HiQ) tuned for DAPI (excitation 370 nm, emission 450 nm) and spectrum orange (excitation 560 nm, emission 590 nm) was utilised, allowing simultaneous detection of DAPI and spectrum orange labels. In addition to the standard Comet parameters, measured as described above, the number and location of hybridisation spots within each Comet was also recorded. Fifty cells were analysed from each slide and measurements from duplicate slides were averaged. The mean of three independent experiments was used to generate each data point.
3. Results

Results obtained from the Comet assay are shown in Fig. 1. With MMC treatment alone, as expected, there were no significant trends in any of the Comet parameters measured. The data shown for Mean % Tail DNA (Fig. 1a) was representative of typical results for other Comet parameters such as Tail Moment. However, when 5 Gy γ-radiation was administered post-lysis to provide a ceiling level of strand breakage, a clear dose response to MMC was observed (Fig. 1b). DNA containing radiation-induced strand breaks is prevented from migrating during electrophoresis due to the accumulation of cross-links, accounting for the relative decrease in Mean % Tail DNA with increasing MMC dose. As MMC-induced cross-links were allowed to repair for up to 24 h prior to irradiation, the radiation-induced damage in the DNA became identifiable as a Comet tail. The relative % Tail DNA value increased with increasing repair time until at 24 h repair, cells displayed comparable levels of radiation induced damage to that shown by cells which received radiation only, indicating that most cross-linking damage had been repaired (Fig. 1c). The representative images in Fig. 2 illustrate how the radiation-damaged DNA was increasingly allowed to migrate to form a larger Comet tail as the time allowed for cross-link repair increased.

To assess the extent of DNA migration induced by strand breakage, a γ-radiation dose response experiment was performed from 0 to 10 Gy. A clear dose response was observed with a 10% increase in Mean % Tail DNA between 0 and 2 Gy (Fig. 3a). Data for various Comet parameters obtained using the Comet–FISH assay compared favourably with data obtained using the Comet assay alone. In the case of the Mean % Tail DNA, values are expressed as percentages relative to damage induced by 5 Gy γ-radiation, indicated by the line at 100%. The Mean % Tail DNA value obtained for 5 Gy was 23.99 ± 1.84. The mean control value was 6.88 ± 0.47. The mean control value obtained for each graph three independent experiments were performed to generate each data point. Error bars shown are ±S.E.M.
Tail DNA measurement, the DAPI stain used during the Comet–FISH protocol provided equal or greater sensitivity than standard Comet assay ethidium bromide stain for a selection of control and repair slides (Fig. 3b).

Data from Comet–FISH experiments also demonstrated a clear dose response to MMC. The percentage of cells showing Comet tails decreased significantly as MMC dose was increased up to 50 μg/ml, since the increased cross-linking prevents DNA migration (Fig. 4a). A cell was considered to have a Comet tail if its % Tail DNA measurement had a greater value than the Mean % Tail DNA value for control cells, which was 5.78 (± 0.41). Noticeably, the percentage of cells displaying TP53 tail spots also decreased as MMC dose increased up to 50 μg/ml. Moreover, the mean number of spots per cell, and per Comet tail, also decreased markedly up to a dose point of 50 μg/ml (Fig. 4b). There was a small, but still significant, increase in the mean number of spots in the Comet head between 0 and 200 μg/ml.

To demonstrate the repair of overall genomic DNA, the percentage of cells showing Comet tails was calculated at each repair time point following treatment with 50 μg/ml MMC. In this case, a cell was considered to have a Comet tail if its % Tail DNA measurement had a greater value than the Mean % Tail DNA value for control cells in these experiments, which was 6.48 (± 0.73). An increase in the percentage of cells showing a Comet tail is observed over repair time as cross-links are repaired and radiation-induced breaks are allowed to migrate (Fig. 5a). Fig. 5a also demonstrates the percentage of cells showing TP53 hybridisation spots in the Comet tail. The percentage of cells showing tail spots is significantly increased during the first 4 h and is also notably faster than the average repair rate of the overall genome (as indicated by the %
Fig. 4. Dose response to MMC measured by the Comet-FISH Assay. (a) Effect on the percentage of RT4 cells showing levels of Tail DNA >5.78% (mean control value) and the percentage of cells with TP53 hybridisation spots in the Comet tail following 1 h treatment with 0, 5, 50 and 200 μg/ml MMC. As dose increases up to 50 μg/ml, the percentage of cells showing Comet tails and TP53 hybridisation tail spots decreases significantly. (b) Analysis of the mean number of spots per Comet and per Comet tail for cells at each dose point. As MMC dosage increases up to 50 μg/ml, the number of spots observed in both the whole Comet and in the Comet tail is significantly decreased (for each graph three independent experiments were performed to generate each data point. Error bars shown are ±S.E.M.).

From 4 to 16 h, the rate of repair of the TP53 gene region and the average overall genomic repair occurs at a similar rate. More rapid repair is again observed in the TP53 gene region relative to the average overall genome from 20 to 24 h, but to a much lesser extent than 0–4 h. Further analysis of TP53 hybridisation spot number and distribution exhibits a similar pattern (Fig. 5b). As repair time increases the mean number of spots per Comet is increased significantly between 0 and 4 h and at subsequent repair time points displays a comparable number to that observed for cells irradiated with 5 Gy alone. Similarly, the number of spots in the Comet tails is also significantly increased between cells with Comet tails) during the same time interval.
and 4 h and then again between 16 and 24 h. It is also noticeable that the mean number of spots in Comet heads is significantly decreased between control and cells irradiated with 5 Gy only. A similar decrease occurs between the 16 and 24 h repair time points.

Frequency analysis of TP53 spot distribution in cells from Comet–FISH experiments further demonstrates the variation in spot number following MMC treatment and during the subsequent repair (Fig. 5c). As expected from a polyploid cell-line, the majority of RT4 control cells display 2, 4 or 6 spots, with some exhibiting as many as 10, although it is worth remembering that this result probably includes a small sub-population of mitotic cells. Noticeably, cells irradiated with 5 Gy alone (i.e. no MMC treatment) demonstrate a wide range in the number of hybridisation spots, with some individual cells showing as many as 15 spots. However, cells which were not allowed to repair following MMC treatment \((t=0)\) exhibit a similar pattern to that for control cells, with most cells showing only 2, 4 or 6 spots, despite being irradiated post-lysis. After 4 h repair time, though, the distribution of spot number resembles that of cells irradiated with 5 Gy only, with many cells showing high number of hybridisation spots. A similar pattern is also observed at both 16 and 24 h repair times.

Fig. 5. Repair of MMC-induced cross-links measured by the Comet–FISH assay. (a) Effect of repair time following 1 h treatment with 50 μg/ml MMC on the percentage of RT4 cells showing levels of Tail DNA >6.48% (mean control value) and the percentage of cells with TP53 hybridisation spots in the Comet tail. As repair time increases, the percentage of cells showing Comet tails and TP53 hybridisation tail spots increases, with a particularly significant increase occurring in both cases from 0 to 4 h. (b) Analysis of the mean number of spots per Comet and per Comet tail for cells allowed to repair for up to 24 h following 1 h treatment with 50 μg/ml MMC. As repair time increases, an increased number of spots is observed in both the whole Comet and in the Comet tail, with a marked increase occurring for both cases from 0 to 4 h. Bars for control (untreated) cells and 5 Gy-irradiated (no MMC treatment) cells are also shown for comparison. (c) 3D histogram showing the frequency of TP53 hybridisation spot number in RT4 cells at each repair time (0, 4, 16, 24 h) following 1 h treatment with 50 μg/ml MMC. As repair time increases, an increased number of cells display high numbers of hybridisation spots. Bars for control (untreated) cells and 5 Gy-irradiated (no MMC treatment) cells are also shown for comparison.

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4. Discussion

Since its conception by Ostling and Johanson in 1984 [12], the Comet assay has proven to be a widely used and extremely versatile technique. Several modified versions of the standard protocol have been developed to study a range of cellular response to DNA damage, including apoptosis, genotoxicity and cross-linking among others [1,2,13]. In order to assess the extent of DNA cross-linking using the Comet assay, cells are treated with the cross-linking agent of interest and then DNA strand breaks are subsequently induced using standard agents such as ionising radiation. The level of strand breakage induced is easily measured by the Comet assay and the relative reduction in the migration of DNA containing strand breaks gives an indication of how much DNA cross-linking has occurred. Since the extent of cross-linking will determine how fast the DNA migrates during

![Representative images of cells at different repair time points following MMC treatment illustrate that hybridisation spots are detected in greater numbers, and more frequently, in the Comet tail as repair proceeds, correlating to the increased migration of DNA as cross-links are repaired (Fig. 6).](image-url)
electrophoresis, it is possible to obtain a relative value for the level of cross-linking within a given cell. This approach has been used to a varying degree of success, with some mutagen-induced cross-links proving easier to measure than others [5,14]. Indeed, Merk and Speit [5] reported that their version of the Comet assay was suitable only for measuring DNA–protein cross-links.

To date, all cross-linking studies using the Comet assay have treated cells with the agent of interest and then exposed the cells to a strand-breaking agent immediately prior to placing in lysis buffer. However, our study differs in that we have irradiated the cells after lysis treatment so that the effect of MMC only is demonstrated, since the γ-radiation delivered after lysis can then exert no influence on any cellular processes, protein functions or gene responses. With this approach, we can be confident that overall repair, and repair occurring in selected gene regions, is of cross-links rather than strand breaks. For the purposes of this study we have used 5 Gy γ-radiation to provide a suitable ceiling level of induced breakage against which the effect of MMC cross-linking is measured, as treatment with MMC alone does not generate significant changes in migration (Fig. 1a).

Using this modified version of the standard alkaline Comet assay, our findings show that the accumulation of, and repair of, MMC-induced DNA cross-links can be measured in RT4 bladder cancer cells. Using our post-lysis radiation strategy, a clear dose response to MMC is demonstrated (Fig. 1b). As the dose of MMC is increased, more cross-links accumulate and the migration of radiation-damaged DNA is subsequently retarded. When the cells are allowed to repair cross-linking damage, the DNA containing radiation-induced damage is free to migrate, as demonstrated by increasing % Tail DNA as repair time increases (Fig. 1c). After 24 h the MMC-induced DNA cross-links have been sufficiently removed for the cells to display comparable levels of damage to that shown by cells which received post-lysis radiation only. Representative images of cells, showing increased Comet tails as repair time increases, illustrate these findings (Fig. 2). Our results agree with previous studies utilising alternative techniques which have shown that MMC-induced cross-links take at least 24 h to repair completely in mammalian cells [15,16] and with a previous study utilising the Comet assay [14]. However, in our modified method we can be confident that the damage induced by the post-lysis γ-radiation can have no effect on gene or protein function and is therefore suitable for the meaningful application of FISH and gene repair studies.

Combining the Comet assay with FISH offers an excellent opportunity to determine the exact location of DNA sequences of interest within a given cell. In particular, the Comet–FISH assay seems particularly suited to the study of repair relating to specific gene sequences following DNA damage. Established methods for measuring gene-specific repair are time consuming and complex and results are only interpretable after high levels of damage and over periods of several hours [17–19]. In contrast, the alkaline Comet assay is a relatively simple technique for measuring DNA damage and subsequent repair, whilst remaining accurate and sensitive. However, despite the potential of this technique, few studies have employed the Comet–FISH combination. Previous studies have used the Comet–FISH assay to detect chromosomal damage in response to UV-radiation [20,21], and a recent study in our laboratory demonstrated that the Comet–FISH assay could be used to follow the response of the TP53 gene to γ-radiation [9]. To date, however, this approach has not been extended to cross-linking studies.

In the current study we have shown that the repair of the TP53 gene region in response to MMC treatment can be followed using the Comet–FISH protocol. Significantly, this technique does not adversely affect the Comet data obtained. In fact, using DAPI to stain the Comet DNA would seem to be even more sensitive than the more commonly used ethidium bromide for measuring Mean % Tail DNA, giving on average a 29% increase in Mean % Tail DNA values (Fig. 3b).

In addition to the Comet data generated, we can also examine the number and position of hybridisation spots within the Comet to provide information about damage and repair of the probed region. However, in interpreting Comet–FISH results we must take into account the amount of DNA that can migrate into the tail in response to one strand break, under the conditions of a given Comet assay experiment. We can estimate this as follows. Breaks induced by ionising radiation in mammalian cells are, at the macro-sequence level, essentially random [22]. 1 Gy induces 0.31 breaks per 10^10 Da DNA [23], therefore with an average base pair mass of 622 Da, 2 Gy induces 0.39 breaks per Mb, or
1 break per 2.6 Mb. Furthermore, 2 Gy drives on average 10% of the DNA into the Comet tail (Fig. 3a). Hence, each break drives a region of approximately 260kb into the tail, assuming both strands of the region affected go into the tail; or a region of 520kb, if only the strands with free ends derived from the break migrates (it is still disputed in the Comet community as to which of these assumptions is correct).

These are estimates of the average amount of DNA moving into the tail. We know that DNA from regions closely and extensively associated with the nuclear matrix, such as replicating DNA, does not move into the tail in standard alkaline Comets even though it must be adjacent to free ends [24]. Given the presence of p53 hybridisation spots in the tail after irradiation, that cannot be the case for this gene in this cell type. The probe used is smaller, at 145 kb, than the average distance migrating into the tail per break; so we cannot exclude the possibility that breaks inside the migrating region, but outside the region probed, could give rise to tail spots.

If we assume, for the present, that the migrating distance per break for the region containing the p53 gene is the same as the average figure, we can now consider three possibilities:

(i) If both strands of the region affected go into the tail, but remain so close together as to be indistinguishable by hybridisation, a break within the probed region or the (possibly larger) migrating domain will produce one spot in the tail and remove one from the head. Two or more spots in the tail can only be generated if two or more breaks occur within the migrating region, a low probability event at low doses.

(ii) If both strands go into the tail separately, a break within the probed region will produce three spots in the tail and remove one from the head; a break within the (average 260kb) migrating domain containing the probed region, but not in the region itself, will produce two spots in the tail and remove one from the head.

(iii) If only the broken strand migrates, a break in the probed region will produce two spots in the tail and remove none from the head. Loss of spots in the head is only possible in this case, if two or more breaks occur in opposite strands within the migrating distance, a low probability event at low doses.

Since in cases (i) and (ii) the probed region is approximately half the size of the average migrating domain, we would expect breaks in the migrating region to occur in a roughly equal ratio in or outside the probed region. Therefore, each break should produce about 1 spot in the tail for case (i), or 2.5 for case (ii); reducing the number of head spots by 1 in each case. In case (iii) the probe region is about a quarter of the migrating region; so each break should produce 1.25 tail spots and remove very few from the head.

Fig. 5b provides some insight as to which mechanism takes place in these experiments. At 5 Gy an average region of 260kb should contain a break in approximately 25% of cases (and there would be few regions with more than one break). In cases (i) or (ii), this should lead to a corresponding decrease in the head spots. In fact, the mean number of head spots per cell decreases from 3.08 to 2.28. We can therefore conclude that our assumption of an average size of migratory region around the p53 gene is approximately correct; and that case (iii) is impossible. To distinguish between cases (i) and (ii), we consider that the 0.8 spots lost from the head should in the first case give rise to about 0.8 tail spots, and the second to about 2. In fact, the mean number of tail spots per cell rises from 0.4 to 2.54. It appears, therefore, that case (ii) alone is consistent with the data; both strands of the migrating region go into the tail, but separately, and about half of the damage we see is within the probed area. However, we acknowledge that this can only be definitively proven by the use of strand-specific probes, and of probes covering further regions of the genome.

In the current study, where MMC treatment is followed by post-lysis γ-radiation, it is the number of MMC-induced cross-links that determines the number and position of the spots since the extent of cross-linking determines how far the radiation-damaged DNA can migrate. Fig. 4a helps to explain the reasoning behind this premise. The percentage of cells showing a Comet tail is significantly reduced as MMC dose is increased. In these experiments, a cell was considered to have a Comet tail if its % Tail...
DNA measurement had a greater value than 5.78, which was the Mean % Tail DNA value from control slides. Since all the MMC treated cells receive the same dose of radiation post-lysis, we can assume that the same number of radiation induced breaks are caused in the probed region at each MMC dose point. However, as MMC dose is increased, the percentage of cells showing tail spots decreases significantly because the increased number of cross-links induced prevents the migration of the radiation-damaged DNA (including the probed region). Analysis of TP53 hybridisation spot number further illustrates the effect of cross-linking (Fig. 4b). The mean number of spots per cell, and per Comet tail, is significantly reduced as MMC dose is increased, once again emphasising that increased cross-linking prevents broken DNA in the probed region from migrating.

Cross-link repair in the cell population as a whole can be demonstrated by calculating the percentage of cells showing a Comet tail at each time point (Fig. 5a). A cell was considered to have a Comet tail if its % Tail DNA measurement had a greater value than 6.48, which was the Mean % Tail DNA value from control slides in these experiments. Fig. 5a shows that, with increasing repair time, the radiation-induced damage is allowed to migrate, resulting in more cells having noticeable Comet tails, and therefore quantifiable levels of DNA breakage. This release of radiation-induced DNA damage is also demonstrated by the increased percentage of cells that display TP53 hybridisation spots in their tails as repair time increases (Fig. 5a). Hybridisation spots located in the Comet tail indicate that the probed region lies within, or in the vicinity of, a region of DNA containing a strand break or breaks. Therefore, it would seem reasonable to conclude that MMC-induced cross-links in this region have been repaired, thereby allowing the $\gamma$-radiation-damaged DNA (including the probed region) to migrate during electrophoresis. The data presented in Fig. 5b supports this proposal, since it shows that the mean number of spots per Comet tail increases with repair time. Indeed, this graph also shows that the mean number of spots in the whole cell increases as the repair time increases, particularly during the first 4 h. As discussed above, the same number of radiation-induced breaks are induced in cells at each repair time point, but it is the degree of cross-linking that determines how this radiation-damaged DNA will migrate. Hence, as repair time increases cells display more spots in the whole cell, as well as in the Comet tail, since the MMC-induced cross-links are being repaired, freeing the radiation-damaged DNA to migrate during electrophoresis.

Frequency distribution analysis of TP53 hybridisation spot number also demonstrates how the number of spots per cell increased as MMC repair time increases (Fig. 5c). Immediately after MMC treatment ($t = 0$) the spot frequency distribution is similar to that for control cells since the MMC-induced cross-linking prevents the DNA (including the probed region) from migrating. However, at 4, 16 and 24 h the pattern resembles that of cells exposed to 5 Gy radiation alone, with many cells showing high numbers of hybridisation spots. The representative cell images in Fig. 6 further emphasise the release of $\gamma$-radiation-damaged DNA as cross-link repair time following MMC treatment increases, as evidenced by increasing Comet tails and by the increased frequency of spots in the Comet tail.

It is noticeable that the release of TP53 hybridisation spots into the Comet tail is most rapid during the first 4 h repair following MMC treatment, implying that cross-link repair in this region has been mostly repaired within the first 4 h (Fig. 5a). TP53 tail spots were observed in 26% of the cells at 0 h, rising to 62% at 4 h with only an additional 20% of cells having tail spots by 24 h. It is also worth noting that the rate of TP53 spot release into the Comet tail is considerably different from the rate of release of overall DNA during the first 4 h (Fig. 5a). Likewise, Fig. 5b shows a significant rise in both the mean number of spots per cell and per Comet tail from 0 to 4 h repair, signifying that the probed region is sufficiently free of cross-links after 4 h to migrate appreciably during electrophoresis. Similarly, the spot frequency distribution graph demonstrates that the number of spots per cell changes dramatically between 0 and 4 h (Fig. 5c). At 0 h the spot distribution profile is comparable to control cells, as the DNA is prevented from migrating due to the MMC-induced cross-linking, despite containing radiation-induced strand breakage. However, after only 4 h repair, the spot distribution pattern is similar to that for cells exposed to 5 Gy radiation only, indicating that the cross-links have been repaired sufficiently to allow the probed region to migrate in a comparable manner.
Given these observations, it is tempting to speculate that this rapid early repair reflects the occurrence of transcription-coupled repair associated with the actively transcribed TP53 gene and this raises the likelihood that the TP53 gene region may be preferentially repaired in comparison to the overall genome. Previous studies have suggested that the TP53 gene is preferentially repaired in comparison to the overall genome in response to UV [25,26] and γ-radiation [9], and it is feasible that a similar preferential repair of the TP53 gene occurs in response to MMC. Indeed, this proposal is given further credence by the fact that some studies have already shown that other actively transcribed genes are preferentially repaired following DNA damage by other cross-linking agents [27,28]. However, this suggestion must remain speculative at present. As mentioned above, we must take into account damage and repair occurring in the vicinity of a probe sequence. To address this issue, ongoing work in our laboratory is focussing on the development of a multicolour Comet–FISH assay, whereby different gene-specific probes of equal size will be hybridised to cells on a single slide. In this way, a direct comparison of repair rates can be made between different gene sequences within an individual cell.

In conclusion, we submit that the modified application of the Comet assay outlined in this study offer increased potential for studying the action of cross-linking agents. The extent of DNA cross-linking alone can be established and the repair mechanics of cross-link damage over time can be closely monitored to ascertain precisely the time point at which cross-links are repaired. Even more promisingly, the Comet–FISH technique simultaneously allows hybridisation of probes to selected gene sequences and is therefore highly suitable for studying the repair of DNA cross-links in specific gene regions in comparison to the overall genome. Indeed, this approach offers a unique opportunity for determining if a repair hierarchy exists between specific gene regions in individual cells in response to DNA damage.

References


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