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Comet–FISH using peptide nucleic acid probes detects telomeric repeats in DNA damaged by bleomycin and mitomycin C proportional to general DNA damage

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For the optimal use of anticancer drugs a knowledge of the whole spectrum of side-effects is required. A potential hazard, so far only scarcely investigated, is uncontrolled effects of drugs such as bleomycin (BLM) and mitomycin C (MMC) on telomere shortening in non-cancerous tissues of the treated person. For the first time, directly labelled telomere-specific peptide nucleic acid (PNA) hybridization probes were applied in comet–FISH to detect DNA fragmentation on an intermediate scale. The effects of BLM and MMC were measured in peripheral blood cells of three human volunteers, following *ex vivo* incubation. Fragmentation of telomeres and subtelomeric regions was highly specifically detected by the comet–FISH assay, a combination of the comet assay and fluorescence *in situ* hybridization. As a technical detail, the effects of the hybridization procedure have been studied on the level of single comets. Image analysis before and after the hybridization process reveals a small decrease in the detected fragmented DNA, probably due to diffusion of small fragments. It could not only be shown that both drugs actually induce breaks in telomere-associated DNA, but also that the comet–FISH technique, as a quantitative approach, is a useful tool for the detection and evaluation of the role of sequence-specific DNA damage after mutagenic action. The breakage frequency for DNA of or adjacent to telomeric repeats was found to be proportional to that of the total DNA, which hints at random induction of DNA breaks by BLM and MMC. In terms of therapy, the results indicate that no over- or under-proportional effects on telomeres of BLM or MMC need be expected.

Introduction

By their specific repeat structure and their ability to form T-loops (Griffith *et al*., 1999), telomeres not only build up and stabilize the ends of chromosomes (Day *et al*., 1993; Knight and Flint, 2000) but, under normal conditions, also protect chromosomes from natural damage. Telomere erosion or loss, on the other hand, destabilizes the human genome and is an early event in DNA damage-induced apoptosis (Ramirez *et al*., 2003). If chemical mutagens, in particular cytostatics, were able to erode telomeres, this would interfere with the natural processes of cellular senescence and/or malignant transformation. Since it is known that the sub-telomeric chromosomal regions contain genes in high density, a knowledge of the susceptibility of these chromosomal regions to breakage is of general interest.

First reports on the action of ionizing radiation indicated telomeres as points of mutagenic attack (Slijepcevic *et al*., 1998; Boei *et al*., 2000). In Chinese hamster cell lines (CHO and CHE) radiomimetic drugs have been shown to induce telomeric damage involved in chromosome breakage and recombination (Bolzan *et al*., 2001). Telomere shortening has recently been reported in patients undergoing chemotherapy (Schroder *et al*., 2001; Lee *et al*., 2003). Telomere breakage sensitivity is also interesting, since DNA structure is known to influence DNA damage and repair mechanisms. These have mainly been studied up to now following radiation-induced damage. In addition, telomeric repeats in damaged DNA can also be considered as signalling breaks in the adjacent gene-rich subtelomeric DNA.

One way to measure sequence specific DNA fragmentation on an intermediate scale (10–800 kb) is the comet–FISH technique. This assay is a combination of the comet assay with fluorescence *in situ* hybridization. Hybridization is performed on electrophoretically separated DNA of a single cell, embedded in agarose. Up to now several hybridization procedures have been described, including those of DNA whole chromosome painting probes (Rapp *et al*., 1999, 2000), gene-specific probes (McKelvey-Martin *et al*., 1998; Schaeferhenrich *et al*., 2003) and centromere probes. Due to the limited hybridization efficiency on agarose embedded cells detection has up to now been dependent on signal enhancing steps, such as antibody cascades or enzyme-enhanced reactions. The application of peptide nucleic acid (PNA) probes has so far only been discussed theoretically as a possible way to overcome this limitation. The use of telomere-specific PNA probes now allows a highly sensitive and reliable detection of telomeric DNA. Therefore, it was considered to also be suited to the detection of damaged DNA closely associated with telomere repeats in 'comets'.

A further question that has not been addressed up to now is whether the hybridization procedure alters the results of overall DNA damage. Since this technique contains several washing steps and high temperature stringency washes it is possible that small fragments are lost from the comet tail and therefore total DNA damage is underestimated.

As mutagens we used two classic cytostatics: the radiomimetic bleomycin (BLM) and the recombinogen mitomycin C (MMC). The antibiotic bleomycin is an S phase-independent radiomimetic antitumoral agent with unique genotoxic properties (Povirk, 1996). The drug is a free radical-based DNA-damaging agent which induces a mixture of strand breaks and abasic sites by highly specific, concerted free radical attack on deoxyribose moieties in both DNA strands. Anderson

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et al. (1997) mentioned that BLM is known to react through oxygen radical mechanisms. A major determinant of BLM-induced damage is the permeability of the cell membrane and the presence of BLM hydrolase, which inactivates BLM. On the other hand, MMC was selected because it mainly induces DNA–DNA interstrand crosslinks (Yang and Wang, 1999) and has been used in previous comet assay as well as comet–FISH studies. Although comet assay results for MMC-treated cells are difficult to interpret, since DNA fragmentation is overlaid by DNA crosslinking effects, we have included this drug to compare the telomere comet–FISH data with recently published data on single copy genes (McKenna *et al*., 2003). It was shown that a concentration 100–800 μM MMC added to human whole blood led to an increase in DNA migration in the comet assay (Pfuhler and Wolf, 1996). As found by McKenna *et al*. (2003) with comet–FISH in RT4 human bladder cancer cells, the number and localization of *TP53* hybridization spots per cell and per comet tail decreased significantly as MMC dose was increased. The authors suggested that this was due to increased crosslinking, which prevents DNA migration.

Materials and methods

Sample preparation and treatment

Human blood was obtained from three healthy, female, non-smoker volunteers 22, 23 and 25 years old. Blood was treated for 1 h with BLM (Hexal AG, Germany) or for 2 h with MMC (from *Streptomyces caespitosus*; Sigma-Aldrich) at 37°C, both at at least three final concentrations. The BLM and MMC concentrations were chosen according to literature data to result in a dose–response curve with minimal cytotoxic effects (Pfuhler and Wolf, 1996; Buschini *et al*., 2002).

Comet assay

The comet assay was performed in its alkaline version following the protocol of Singh and Tice (Singh *et al*., 1988; Tice *et al*., 2000). In short, 90 μl of cell/agarose suspension (containing 8 μl of whole blood with 0.9% low melting point agarose, Type VII; Sigma-Aldrich) were distributed onto frosted microscope slides (Labcraft, London, UK) precoated with: ground layer agarose (Type II; Sigma-Aldrich); 1.0% normal melting point agarose in phosphate-buffered saline (PBS) (100 mM phosphate) and 400 μl of middle layer agarose; 1.0% normal melting point agarose in PBS (100 mM phosphate). After the agarose solidified, slides were immersed in cold lysis solution (10 mM Tris-(hydroxymethyl)-aminomethane, pH 7.5, 100 mM Na_2EDTA, 2.5 M NaCl, 1% Triton X-100, pH 10) for 60 min at 4°C. The slides were placed in an electrophoresis chamber containing alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.1, 4°C) for DNA unwinding. After 20 min, the current was switched on and electrophoresis was carried out at 1.25 V/cm, 300 mA for 25 min. The slides were removed from the electrophoresis chamber and washed once for 10 min with neutralization buffer (0.4 M Tris–HCl, pH 7.5, 0.08 M Tris base, pH 7.2). The slides were stained with SybrGreen (diluted 1 μl/ml and 30 μl/slide). All of the steps of the comet assay were conducted under dim light. The experiments were reproduced independently at least twice.

Total DNA damage was microscopically quantified using a Zeiss Axioplan microscope, equipped with an HBO 50 and appropriate filter sets for FITC detection (Zeiss no. 9). Images were recorded using an intensified video camera (Variocam; PCO, Germany) and were captured to a PC running Komet 4 software (Kineticimaging, UK).

Comet–FISH

For comet–FISH the slides were prepared as described above, but the staining was omitted. Before hybridization the gels were stored for at least 3 days in absolute ethanol at 4°C for dehydration. The gels were rehydrated in H_2O for 15 min and subsequently the DNA was denatured by incubation in 0.5 M NaOH for 25 min. The denatured DNA was immediately dehydrated in an ethanol series (75, 80 and 95%, 5 min each) and the gels were carefully air dried until all ethanol had evaporated. PNA probes (Telomere PNA FISH Kit/Cy3; Dako Cytomation, Denmark) were used according to the instructions of the manufacturer, but since thermal co-denaturation is not possible with agarose gels the hybridization probe was prewarmed to 60°C and 10 μl were applied to an area of ~20 × 20 mm. The gels were sealed with a plastic coverslip and the slides placed in a humidified chamber at 37°C overnight. The next day the slides were placed at room temperature for 30 min before they were placed in 1× rinse solution supplied with the PNA probes, in order to facilitate removal of the plastic slides. Then the slides were transferred for 2.5 min to a staining jar containing prewarmed wash solution (from the PNA Kit) at 65°C, without agitation. The slides were then quenched in cold 1× phosphate-buffered detergent (PBD). For counterstaining the slides were embedded in 1:2000 diluted SybrGreen (Molecular Probes, Germany) including 50% antifade.

The number of telomere signals and the localization of the signals (comet head or comet tail) were additionally recorded for each cell. Cell numbers scored for comet–FISH ranged from 50 to 100 cells/slide.

Image analysis and comet–FISH evaluation

From the total DNA profile, the Komet 4 software package calculates the head to tail border, according to the maximum intensity. This line was used to count the hybridization spots semi-automatically in the head and tail regions. For each comet the total DNA damage (expressed as a percentage of DNA in the tail) was correlated with the telomere-associated damage expressed as a percentage of telomeres located in the tail.

To control hybridization efficiency, 3-dimensional laser scanning microscopy was performed using a Zeiss LSM 510, equipped with argon ion and helium/neon lasers. This analysis revealed up to 90 hybridization signals (mean 84 ± 7, $n = 5$) per comet with the telomere probe. Since the normal human karyotype contains 92 telomeres, the percentage of telomeres in the image plane in epifluorescence imaging was calculated. From the three replicates per concentration a minimum of 20 comets per slide were further analysed. The 50 comets with the highest hybridization efficiencies were selected for the statistical analysis.

Repeated scanning analysis

To study the effect of the hybridization procedure at the level of the single cell we stained the slides after the comet assay, imaged several comets and relocated the same comets after the hybridization process, using marks engraved on the slides. Then the images were analysed with the Komet 4 software package and the total intensity as well as the percentage DNA in the tail were compared.

Results

Visual analysis of the induced comets under the microscope revealed distinct differences in shape and length between the applied mutagens: BLM induced longer and thinner comet tails than did MMC. The length of the former, in addition, showed a clear dose dependence. MMC induced rather short and more voluminous tails with a less clear dose dependence. While at lower doses of MMC the length of the comets increased in a dose-dependent manner, the highest dose induced shorter comets on average than the previous one. These effects, documented as the results of the quantitative comet image analysis, are summarized in Table I.

Dose–response characteristics for total DNA damage and damage associated with telomeric sequences

In order to study the effects of BLM and MMC treatment on DNA connected with telomeres we elaborated the dose–response relationship for total DNA fragmentation and telomere signals found in the migrating DNA portion (comet tail).

Control nuclei show a majority of only weakly damaged comets, with a low number of telomeres outside the head. Additionally, few cells exist with a high percentage of telomeres in the tail ($>60\%$) and moderate total DNA damage.

Figure 1A shows the DNA damage induced by BLM together with the percentage of detected hybridization signals located in the comet tails. Both curves show a steep dose-dependent increase, which is linear for doses up to 25 IU/ml BLM. At higher doses (100 IU/ml) the effect is only marginally increased. Therefore, the total data can be described by a exponential rise to a maximum fitted function. The proportion of telomeric signals in comet tails exceeds the relative amount of total DNA in the tail in all examined cases, but parallels the overall tendency of total DNA damage. The values for total

Table I. Dose–response characteristics and experimental conditions for bleomycin and mitomycin C treatment (n = 120)

Treatment	DNA in tail (%) (mean ± SD)	Comet–FISH (n)	Telomeric signals per comet in the image plane (%) (mean ± SD)	Telomeric signals in tail (%) (mean ± SD)
BLM				
Control	7.5 ± 1.9	50	16 ± 5	17.8 ± 5.2
12.5 IU	24.4 ± 3.8	50	21 ± 10	41.3 ± 13.7
25 IU	37.6 ± 3.5	50	22 ± 11	49.5 ± 1.0
100 IU	38.2 ± 3.3	50	21 ± 5	52.1 ± 19.2
MMC				
Control	5.9 ± 3.4	50	12 ± 5	15.5 ± 12.8
25 μg/ml	22.8 ± 1.8	50	13 ± 5	25.5 ± 16
50 μg/ml	31.0 ± 8.6	50	15 ± 6	44.6 ± 13.2
100 μg/ml	22.5 ± 10.3	50	20 ± 9	29.1 ± 17.5

Comet assay and Comet–FISH results with PNA telomeric probes are listed. Total DNA damage is expressed as DNA in tail (%) (mean ± SD). Telomeric signals per comet in the image plane (%) (mean ± SD) represents the mean detected number of hybridization spots per image (for details see text). Telomere-specific damage is expressed as Telomeric signals in the tail (%) (mean ± SD).

damage together with the percentage of telomeres found in the tail for the BML treatment are summarized in Table I. Typical sample micrographs are shown in Figure 1C–E, representing comets with increasing total DNA damage and increasing numbers of telomeres in the tail. At this point it must be mentioned that only a fraction of the total number of telomeres are visible if 2-dimensional epifluorescence microscopy is used for analysis. The real hybridization efficiency was controlled by 3-dimensional laser scanning microscopy and in a small number of comets the telomere signals were counted. An average spot number of 84 signals/comet was found, but only a fraction of them are visible in epifluorescence images. The mean 'hybridization efficiency' measured is given in Table I for each specimen, expressed as Telomeric signals per comet in the image plane (%) (mean ± SD). This value is calculated as number of signals in the recorded image divided by 92.

In contrast to BLM, MMC treatment leads to decreased DNA migration at doses >25 μg/ml. The data are plotted in Figure 1B. Similar to the findings for BLM, the percentage of telomeres found outside the head region exceeds the percentage of total migrated DNA. The kink in the dose–response curve at MMC concentrations >25 μg/ml represents the known crosslinking effect of MMC. The percentage of

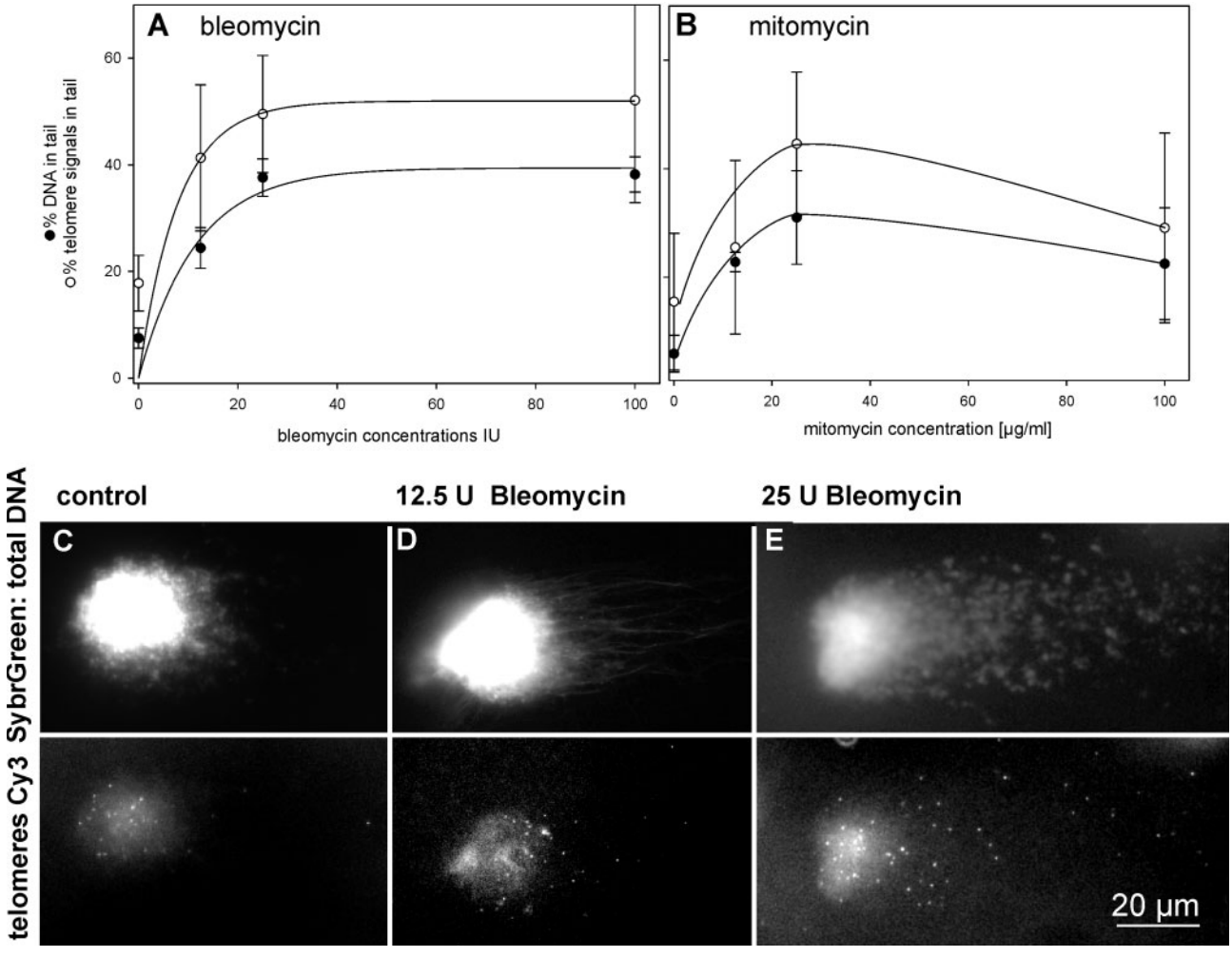

Fig. 1. Dose–response relationship between the total DNA in the tail and the telomere-containing fragments expressed as 'percentage of telomeric signals in tail'. (**A**) After bleomycin exposure. (**B**) After mitomycin exposure. The percentage of telomere signals (open circles) in the tail in both cases exceeds the relative amount of total DNA (filled circles) in the tail, but parallels the slope of tail DNA. Mean values ± SD are plotted. (**C–E**) Sample images of comets exposed to 0 (C), 12.5 (D) and 100 IU (E) bleomycin. The upper row shows the total DNA stained with SybrGreen, whereas the lower row shows Cy3-labeled telomeres.

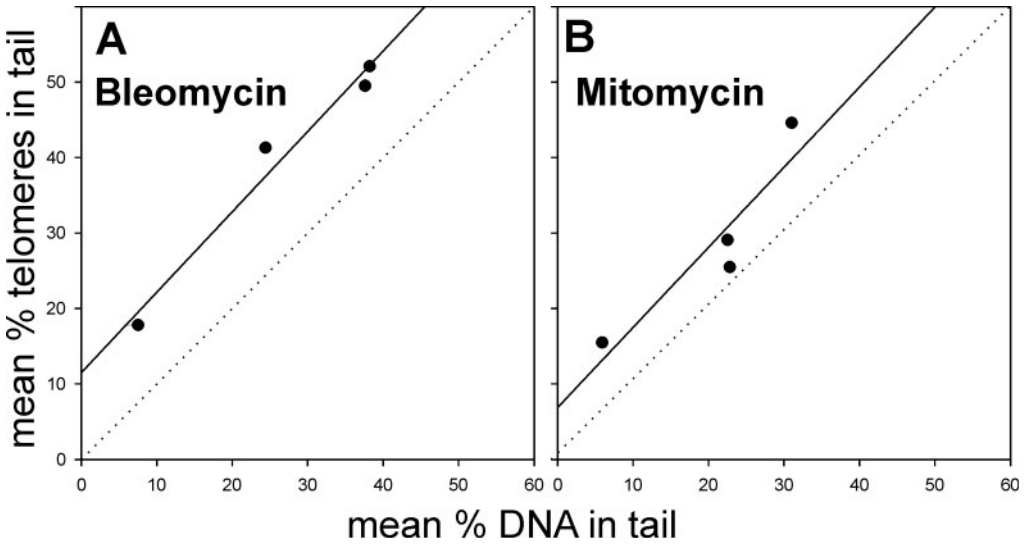

Fig. 2. Percentage of telomeric signals in the comet tails as a function of the percentage of total DNA in the tail for human peripheral blood cells. The solid straight lines are obtained by linear regression analysis of the mean values. The resulting slopes are not significantly different from 1. The shift of the lines towards higher than statistical (dotted lines) percentages of DNA in the tails indicates a drug-independent increased fragility of DNA associated with telomere repeats.

damaged telomeres follows the percentage of total damaged DNA over the whole dose range examined. The detailed data for the MMC measurements are also given in Table I.

The mean percentage of telomeres found in the tail [Telomeric signals in tail (%)] was plotted against the mean value of total DNA in the tail [DNA in tail (%)] in Figure 2. Both treatments (BLM and MMC, Figure 2A and B) led to a linear dependency between DNA in the tail and percentage of telomeres found in the tail, with a slope of 1.07 for the BLM treatment and of 1.03 for the MMC treatment. These values are not significantly different from a slope of 1.0 that would be expected for a random distribution of DNA damage.

Controls for the MMC treatment are comparable to the controls for BLM, with the difference that the absolute values for telomere-specific damage are slightly lower for the first treatment. Again, treatment with MMC led to increased total DNA damage as well as to increased telomere damage. With increasing MMC doses the telomere friability (expressed as a percentage of telomeric signals found in the tail) seems to be independent of total DNA damage.

Comparison of the comets before and after hybridization

To investigate the effects of the hybridization procedure on measurement of total DNA damage, comets were stained before and after the hybridization procedure and were relocated for analysis of individual comets. Figure 3 shows the data obtained from the analysis of 60 comets at three different concentrations of BLM and an additional control, as well as from MMC-treated cells. The percentage of DNA in the tail decreases during hybridization, possibly due to the washing steps and diffusion of the fragmented DNA. The average decrease was found to be 19.8 ± 12.6%, but the loss of DNA was larger in heavily damaged cells compared with weakly damaged ones.

Although the absolute values for comet intensity changed during the hybridizations, the percentage of DNA in the tail changes only slightly and the dose–response relationship was not dramatically altered by the hybridization procedure. Also, the total intensity of the comets decreases during hybridization.

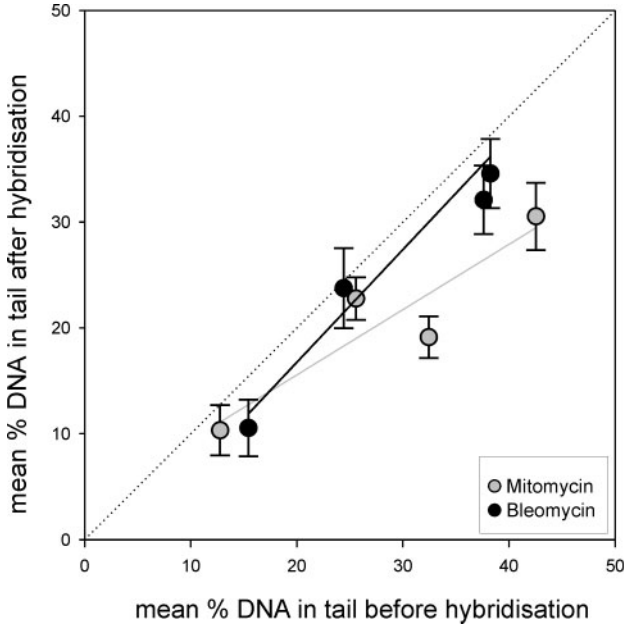

Fig. 3. Comparison of the measured DNA damage before and after the hybridization procedure. Individual comets were relocated and the total DNA damage was quantified as '% DNA in tail'. The hybridization process leads to a general decrease in the detected damage compared with the theoretical dotted line that represents zero effects. Nevertheless, the dose–response relationship was preserved after the hybridization procedure.

Discussion

The importance of the comet–FISH technique has previously been shown with respect to the detection of repetitive (alphoid) DNA sequences (Santos *et al*., 1997; Fernandez *et al*., 1998), of specific chromosomes using painting probes (Santos *et al*., 1997; Fernandez *et al*., 1998; Rapp *et al*., 2000) and specific gene probes (Santos *et al*., 1997; Fernandez *et al*., 1998; McKelvey-Martin *et al*., 1998; McKenna *et al*., 2003; Schaeferhenrich *et al*., 2003). All these studies used DNA

probes which were dependent on signal enhancing steps. In the present paper telomere repeat-specific PNA probes were used for the first time in combination with single cell gel electrophoresis, which allows direct detection of the signals in comet heads and tails. Their high specificity is reflected in the very small but brilliant signals of pure $(TTAGGG)_n$ repeats in the comet heads and tails (up to their utmost end).

In our view the different molecular mechanisms of action of the agents examined are reflected in the outcome of our experiments: BLM and MMC induced comets of different shapes and sizes in the comet assay. These differences were also reflected in the distribution of telomeric repeat signals in the heads and tails of the comets: While after treatment of the cells with BLM sharp telomeric signals were found spread over the whole tail, a considerable number remain close to or within the head in the shorter comets after MMC treatment.

These findings confirm previous data obtained by other authors with these standard mutagens. While Anderson *et al*. (1997) suggested that BLM can react through oxygen radical mechanisms, MMC mainly induces DNA–DNA interstrand crosslinks (Yang and Wang, 1999). Pfühler and Wolf (1996) found an increase in DNA migration in the comet assay when MMC was added to human whole blood at a concentration of 100–800 μmol. However, McKenna *et al*. (2003), using the comet–FISH technique in RT4 human bladder cancer cells, reported a decrease in signals in the tail with increasing MMC dose. They suggested that the effect of reduced DNA, as well as hybridization signals in the tail, was due to increased crosslinking, which prevents DNA migration. This was supported by data on the role of interstrand crosslinks in the reduction in free DNA fragments, mainly detected under alkaline conditions, presented by Klaude *et al*. (1996) and Merk and Speit (1999).

Our investigations have shown that the hybridization procedure has a detectable effect on the measured total DNA damage, but the influence is small. This may be attributed to two different processes. First, DNA might be lost, especially from the tail, by diffusion and therefore is missed in the analysis after hybridization. On the other hand, the denatured DNA is possibly not completely rehybridized and therefore partly single-stranded. Since the dyes used are intercalating dyes, they are less effective if some of the DNA in the comets is single-stranded. These findings will result in an underestimation of the detected damage. The overall dose–response curve for total DNA damage, however, is preserved throughout the hybridization steps. This observation led to a recommendation of parallel specimen preparation and analysis for total DNA damage and for hybridization.

The detection of DNA breakage within telomeres by the comet assay is limited by the size of DNA fragments detectable by this technique (10–800 kb) (Rapp *et al*., 2000). Since estimates of telomere length in human peripheral lymphocytes vary between 5 and 15 kb (Perner *et al*., 2003), fragments of these segments will be detected only in exceptional rare cases and cannot be differentiated from breaks in telomere-adjacent DNA. However, the finding of signals in the comet tails is equally important in that it reflects breaks in the telomere-adjacent sub-telomeric sites, with their high gene density.

A comparison of total DNA damage with telomere-specific damage results in non-significant differences for the two drugs tested. While the percentage of telomeres in the tail exceeds the total DNA damage for all concentrations tested, the proportion of telomere-specific damage to total damage remains constant. This leads to the conclusion that the damage induced by MMC and BLM is random with regard to telomere repeats. Since the damage induced by BLM and MMC is dependent upon the distribution of the drugs inside the nucleus, a diffusion-driven distribution would be expected which would cause this randomness. Both mutagens show this behaviour under the test conditions. While BLM-treated cells show higher levels of DNA in the tail compared with MMC-treated ones, the percentage of telomeres in the tail is reduced. The fact that in control cells more telomeres are outside the comet head than total DNA probably reflects the intranuclear distribution of the telomeres in the nuclear periphery (Santos *et al*., 1997).

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