Comet-assay in combination with PNA-FISH detects mutagen-induced DNA damage and specific repeat sequences in the damaged DNA of transformed cells

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Abstract. The Comet-assay was applied to three transformed cell lines (HT1080, CCRF-CEM line and CHO) which were treated with the cytostatics bleomycin (BLM) or mitomycin C (MMC). In addition, PNA probes for the telomere repeat $(TTAGGG)_n$ were used for detection of telomeric DNA sequences in the damaged DNA. Data were compared with previously obtained results from peripheral leukocytes. The amount of migrating DNA increased in all cell types in a dose-dependent manner after BLM exposure. CHO cells reacted sensitively at low doses of the mutagen, and leukocytes had the highest dose-related effect up to 25 IU/ml which, however, did not further increase. A rather linear dose response characterized the HT1080 cells, the effect was lowest for the CCRF-CEM cells. While MMC at lower doses increased the percentage of migrating DNA in a dose-dependent manner, the higher doses induced shorter comets, on average, than the lower ones in all cell lines. With PNA-Comet-FISH obvious differences were found between the studied cell lines with respect to quantitative head/tail distribution of telomeric signals after BLM exposure. A large number of signal spots of various sizes were found in CHO cells, very small signals could be detected in the comets of both neoplasia cell lines. Dose-dependence of telomeres in the tail was most pro-nounced in CCRF-CEM and normal leukocytes, less in HT1080. The steepest dose-related increase of telomeric signals in the tail was found in CHO cells. The ratio between the migrated DNA and the telomeric signals in the tail varied distinctly between the examined cell types from 3:1 to 1:1. Taken together, Comet-FISH can detect mutagenic effects on specific DNA sequences. This may be of high practical value if amplified DNA sequences will be addressed by those examinations in future.

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Introduction

The Comet-assay (single cell microgel electrophoresis) was originally developed for the detection of mutagen-induced DNA damage (1-4) such as by electric field DNA fragments or loops are extruded from agarose-embedded nuclei of single cells. As many cytostatic agents act via damaging of DNA it could be of some interest in the control of side effects of cancer therapies (5-8) and in control of therapy resistance (9-11). New horizons were recently opened to this technique by application of specific fluorescent *in situ* hybridizing (FISH) probes for searching specific DNA damage: Up to now several hybridization procedures have been described including those of DNA probes for whole chromosome painting (12,13), or specific genes (14-16).

Telomeres, by their specific repeat structure and their ability to form T-loops (17), not only build up and stabilize the very ends of chromosomes (18,19), 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 of DNA damage-induced replicative senescence, apoptosis and cancer (20-22). If chemical mutagens, and in particular, cytostatics are able to attack telomeres or telomere-adjacent regions, this could interfere with the natural processes of cellular senescence and/or malignant transformation. As it is known that the sub-telomeric chromosomal regions contain genes in high density, knowledge on the susceptibility of these chromosomal regions vs. breakage is also of high general interest. Previous findings using telomeric probes, indeed, pointed to damage in the adjacent sub-telomeric DNA regions (23).

A suitable way to measure sequence specific DNA fragmentation on an intermediate scale (10-800 kbp) is the Comet-FISH technique. It combines the Comet-assay with fluorescence *in situ* hybridization. The hybridization is performed on the electrophoretically separated DNA of single cells embedded in agarose. Due to the limited hybridization efficiency on agarose embedded cells the detection was up to now dependent on signal enhancing steps, such as

antibody cascades or enzyme enhanced reactions. The application of peptide nucleic acid (PNA) probes was so far only discussed theoretically as a possible way to overcome this limitation. The use of a telomere-specific PNA probe now allows a highly sensitive and reliable detection of telomeric DNA (23,24). This type of probe is considered to be suited for the detection of specific DNA damage closely associated with telomere repeats in comets.

For the induction of comets two classic cytostatics, the radiomimetic Bleomycin (BLM) and Mitomycin C (MMC), were used for the present study.

The antibiotic bleomycin is an S-phase independent radiomimetic antitumoral agent with unique genotoxic properties (25): '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 deoxiribose moieties in both DNA strands'. On the other hand, Mitomycin C was selected because it mainly induces DNA-DNA inter-strand cross-links (26).

As it is of particular interest if the telomeric regions of transformed cells react in a way similar to normal human leukocytes, three model cell lines were used in the present study in order to compare total DNA damage and telomere-associated breakage.

The Chinese hamster ovary (CHO) cell line seemed obviously suited because of its two main features, i.e. big interstitial blocks of telomeric repeats in addition to the normal end-standing telomeres (27), and its low repair capacity (28). As human neoplasia lines the fibrosarcoma-derived line HT1080, and the T-acute lymphocytic leukemia line CCRF-CEM were considered to be representative models for the present comparative examinations.

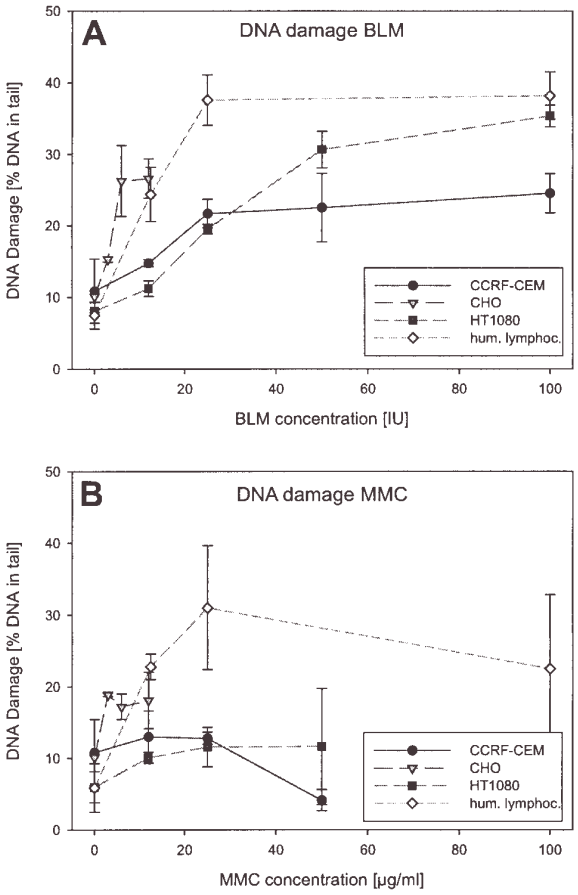

Figure 1. Dose-dependence of DNA damage (expressed as % DNA in tail) induced by Bleomycin (A) and Mitomycin C (B) in three transformed cell lines and normal human peripheral leukocytes (for comparison).

Materials and methods

Cell lines and treatment. The transformed cell lines HT1080 (derived from a human fibrosarcoma), CCRF-CEM (derived from a human T-cell acute lymphocytic leukemia), and CHO (Chinese hamster ovary) were used throughout all experiments. Cells were cultured at 37˚C in RPMI containing 10% fetal calf serum, l-glutamine, and of a standard penicillin/streptomycin solution (1%) up to their use for the experiments. Cells were exposed for 1 h to Bleomycin (Hexal AG, Germany) or for 2 h to Mitomycin C, in at least three final concentrations. The concentrations were chosen according to literature data and our previous experiences (29) in order to result in a dose response curve with minimal cytotoxic effects.

Comet-assay. The Comet-assay was performed in its alkaline version following the protocol of Singh and Tice (1,3). In short, 90 µl of cell/agarose suspension (containing 20 µl of cell suspension 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 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 10.0) for at least 60 min at 4˚C. The slides were placed in an electrophoresis chamber containing alkaline buffer (1 mM Na_2EDTA; 300 mM NaOH, pH 13.0; stored at 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 SYBR-Green (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 two times.

Comet-FISH. For Comet-FISH the slides were prepared as described above, but the staining was omitted (13,29). 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 was evaporated. PNA probes (Telomere PNA FISH kit/Cy3; DakoCytomation, Denmark) were used according to the instructions of the manufacturer. Since thermal co-denaturation is not possible with the agarose gels, the hybridization probe was prewarmed separately to approximately

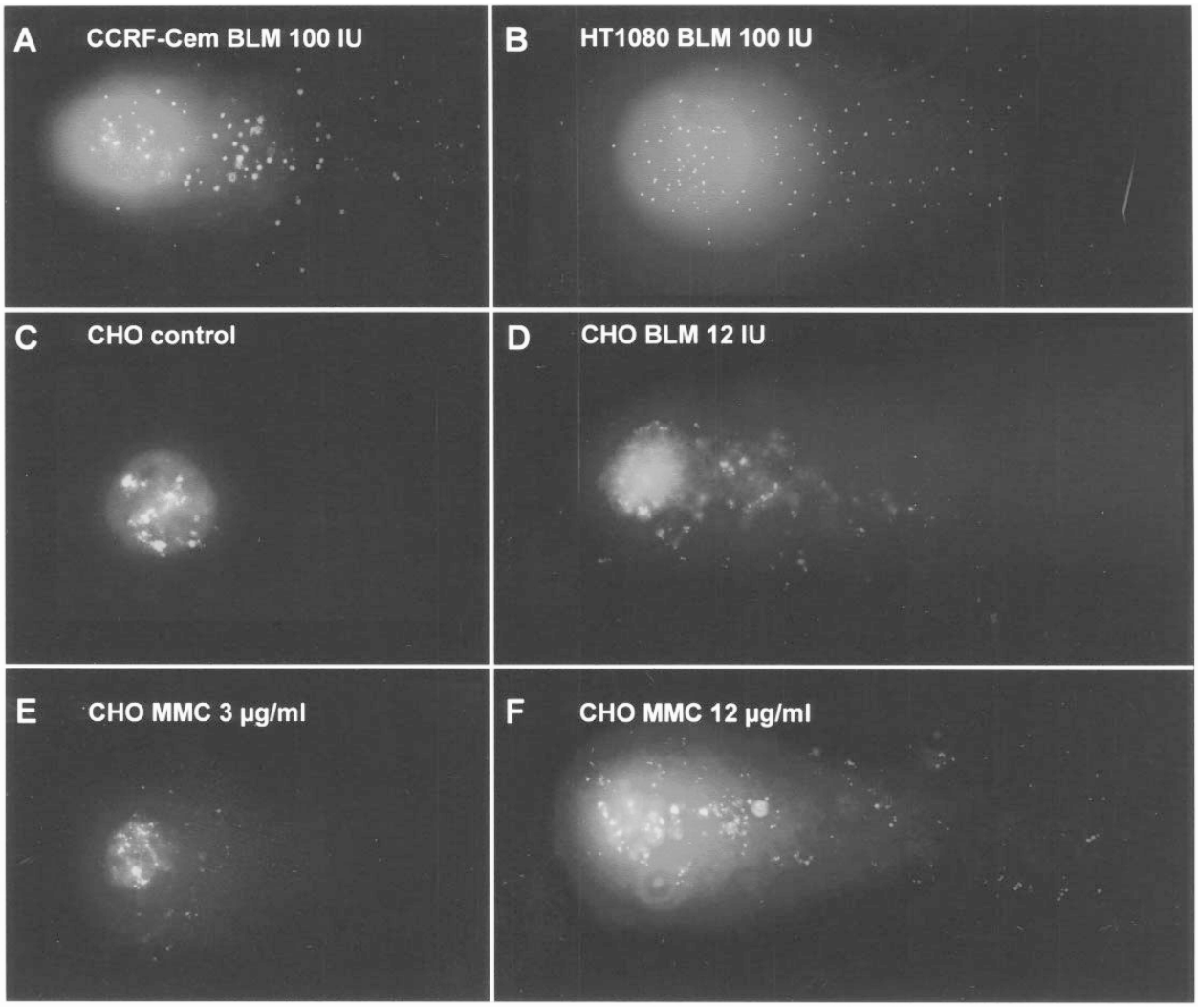

Figure 2. Sample images of the outcome of Comet-FISH in the examined cell lines using telomeric PNA probes for the detection of telomeric repeat sequences and SYBR-green for staining the total DNA (cell type and applied mutagen doses are given in the inserts).

80˚C and 10 µl were then applied to an area of approximately 20x20 mm. The gels were sealed with a plastic coverslip and the slides were 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 rinsed in 1X rinse solution of the kit, in order to facilitate the removal of the plastic slides. Then the slides were transferred for 2.5 min into a Coplin jar with prewarmed wash solution at 65˚C, without agitation. Then the slides were quenched in cold 1X PBD. For counter staining the slides were embedded in diluted SYBR Green (Mobitec, Göttingen) including 50% antifade (Vectashield).

Microscopic evaluation. Global DNA damage was microscopically quantified using a Zeiss Axioplan microscope, equipped with an HBO 50 and appropriate filter sets for detection of Cy3 and SYBR-green. Images were recorded using an intensifying video camera (Variocam, PCO, Germany) and were captured to a PC running the Komet 4 software package (Kineticimaging, UK).

The number of telomere signals and their localization (comet head or comet tail) were additionally recorded for each cell. Cell numbers scored for Comet-FISH ranged from 50-100 cells per slide.

Results

Induction of comets by BLM and MMC was compared between the three examined cell lines and with previous data from experiments on human peripheral blood leukocytes. As shown in Fig. 1A, the amount of DNA in comet tails increased in all cell types in a dose-dependent manner in response to BLM exposure. CHO cells reacted in a very pronounced way to low doses of the mutagen, the highest dose-related effect of ≤25 IU/ml was seen in leukocytes, however, it did not further increase at higher doses. A rather linear dose response characterized the fibrosarcoma cell line while the lowest effect was observed with the T-ALL derived cell line CCRF-CEM. While MMC at lower doses increased the percentage of extranuclear (migrating) DNA dose-dependently, the higher doses induced shorter comets, on average, than the lower ones in all cell lines.

Clear differences between the cell lines could also be observed with respect to the shape of comets induced by BLM and MMC (compare Fig. 2A with E). While BLM induced longer and thinner comet tails than did MMC, the latter produced rather short and more voluminous tails. Moreover, also the cell type examined apparently influenced

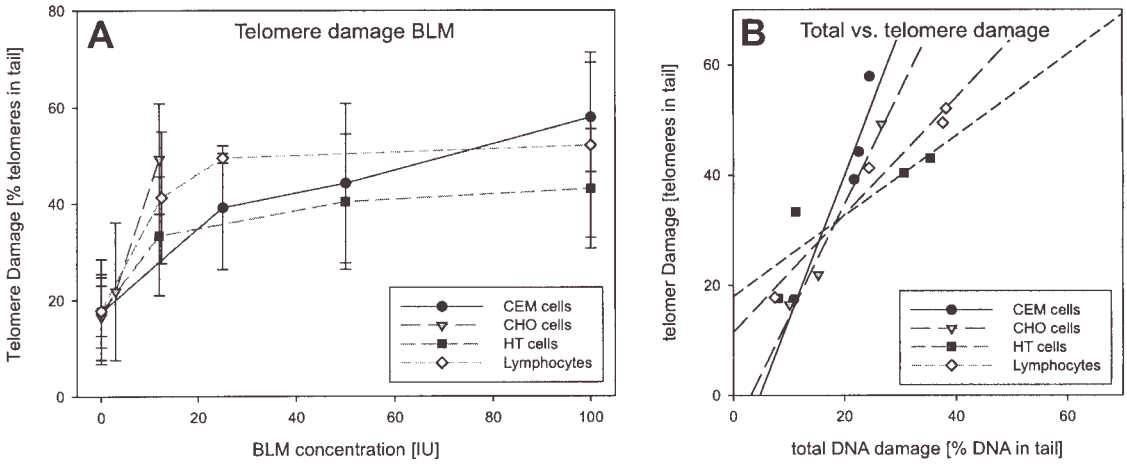

Figure 3. (A) Dose-dependence of the percentage of telomeric signals in the tail (as compared with that of the comet head) induced by Bleomycin in three transformed cell lines and normal human peripheral leukocytes (for comparison). (B) Linear regression analysis of percentage of telomeric signals as a function of the percentage of total DNA in the tail after BLM exposure of the three examined cell lines and normal human peripheral leukocytes. The resulting slopes are different from 1 for CCRF-CEM (2.62±0.55) and CHO (2.06±0.33), close to 1 for leukocytes (1.06±0.13), and for HT1080 (0.73±0.28).

these differences [compare Fig. 2A (CCRF-CEM) or B (HT1080) with D (CHO), both after BLM treatment].

PNA telomere repeat probe readily detected signals in heads and tails of the comets (Fig. 2). By BLM, obvious differences were produced between the studied cell lines with respect to quantitative head/tail distribution of telomeric signals. Signal spots of various, sometimes rather large size were found in comet tails of CHO cells (Fig. 2C-E), a number of distinct, but sometimes also very tiny signals could be detected in the comets of both human neoplasia cell lines (Fig. 2A andB). Evidently, there was a clear dose-dependence of the percentage of telomeres in the comet tail, most pronounced in CCRF-CEM cells and normal leukocytes, less in HT1080 cells. The steepest dose-related increase of telomeric signals in the tail was found in CHO cells (Fig. 3A). The relative amount of DNA and the number of telomeric signals outside the nucleus varied distinctly between the examined cell types (Fig. 3B): In CCRF-CEM cells this was about 2.62±0.55, and in CHO 2.06±0.33, while in HT1080 it was <1 (0.73±0.28) and in normal leukocytes it was equal to percentage of tail-DNA (1.06±0.13). An exemplary experiment with MMC (0-12 μg/ml) in CHO cells similarly yielded a steep dose-dependent increase of the percentage of extra nuclear telomeric signals.

Discussion

DNA damage-induced by the two classical cytostatics bleomycin and mitomycin C could be shown in Comet-assay to occur in the form of DNA extruded from the nuclei (comet tails) in a dose-dependent way also in all examined transformed cell lines of the present study. Nevertheless, there were clear differences with respect to the extent of DNA damage induced by specific agent doses. In addition, as has been described previously (29), clear differences in the shape and size of comet tails were found between BLM and MMC as inducing agent. It was of particular interest that clear differences of mutagen sensitivity could be detected between the studied cell lines. For instance, peripheral leukocytes showed a more distinct response to the cytostatics in terms of DNA damage detected by comet assay than both neoplastic cell lines. The most drastic increase of the amount of damaged DNA induced by very low doses of both, BLM and MMC, was found in CHO cells, the lowest response in CCRF-CEM cells. The main reason for the observed high mutagen sensitivity of CHO probably is the well known repair-deficiency of most CHO cells (28). Thus, the use of the Comet-assay to estimate drug sensitivity of cancer cells (9-11) could convincingly be confirmed by the present findings.

The Comet-FISH technique used in this study has previously been shown to be a potent method for the detection of repetitive (alphoid) DNA sequences (23,24), of specific chromosomes using painting probes (13,23,24) and specific gene probes (4,14,15,23,24). All these studies, however, used DNA probes which were dependent on signal enhancing steps. As first described by our group (29), telomere-repeat-specific PNA probes readily allow a direct detection of the signals in comet head and tails of normal human leukocytes without additional enhancing steps.

In clear dependence on the cell type under consideration, the differences found with respect to total DNA damage were also reflected by the distribution of telomeric repeat signals in the heads and tails of the comets: After treatment of the cells with BLM sharp telomeric signals were found spread over the whole tail, but a considerable number also remained close to or within the head. The latter picture predominated in the shorter comets after an exemplary MMC treatment of CHO cells. These findings were in clear support of the considerations mentioned before on the molecular mechanisms of action of both cytostatics. BLM can react through oxygen radical mechanisms (25) inducing a great number of DNA double strand breaks also within or in the close neighbourhood of telomeric repeats. The induced DNA fragments, therefore, are spreading over the comet tail. In contrast, MMC which mainly induces DNA-DNA interstrand cross-

links (26) in part leads to 'retaining' broken DNA within or in close environment of the nuclear volume 'comet heads'. Pfuhler and Wolf (30) found an increase of DNA migration in the Comet-assay when the mutagen was added to human whole blood at a concentration 100-800 µmol MMC. McKenna *et al* (4) using Comet-FISH in RT4 human bladder cancer cells, however, reported that the number and localization of TP 53 hybridization spots per cell and per comet tail decreased significantly as MMC dose was increased. The authors suggest that this was due to increased cross-linking which prevents DNA migration. This was supported by data on the details of interstrand crosslinks in the reduction of free DNA fragments, mainly detected under alkaline conditions presented by Klaude *et al* (2) and Merk and Speit (31).

One feature which may be responsible for the observed rather high number of telomeric signals of various size in the comets of the CHO cell line is the well known fact that the chromosomes of these cells contain very large interstitial telomeric chromatin blocks (27,32). The latter, apparently, are rather accessible for the attack of DNA damaging agents or ionizing radiation (32). However, the T-ALL line also showed a significantly higher sensitivity of telomeric or telomere-adjacent DNA for fragmentation by BLM than did the total DNA. It remains to be elucidated if the subtelomeric or even telomeric segments of this line show any genomic instability. In contrast, the human fibroblastoma cell line did not differ substantially from normal leukocytes or was even less sensitive with respect to its general response to both mutagens as well as to its fragility of telomeric or telomere-adjacent DNA segments, respectively.

The fragility of telomeric or subtelomeric chromosomal regions under cytostatic exposure, however, does not absolutely give rise to general telomere shortening as could be shown by systematic measurements of telomere length after mutagen exposure (Wick *et al,* unpublished data), because telomerase activity of transformed cells may restore damaged telomeres. For instance, the CCRF-CEM line used in our present experiments clearly shows telomerase activity (Wick, unpublished data).

Two limitations of Comet-FISH analysis of telomeric repeats (29) must also be observed when evaluating the present data on transformed cells: a) Our previous investigations (29) have clarified that the hybridization procedure indeed has a detectable effect on the measured total DNA damage, but the influences are small. The overall dose response for the total DNA damage is preserved throughout the hybridization steps. The loss of fragments in the tail is probably due to the higher diffusion rate of the fragmented DNA. This will lead to an under-estimation of the detected damage. Another influence, not mentioned up to now is that the dyes for total DNA staining (e.g. SYBR-green used here) are specific for double stranded DNA. Denaturation-renaturation during the Comet-FISH procedure can decrease the total fluorescence intensity of the comet by an incomplete renaturation and remaining single stranded DNA tracks. Taken together, this observation led to the recommendation of parallel specimen preparation and analysis for the total DNA damage and for the hybridization, although the effects of the hybridization in our experiments do not significantly alter the dose response for the tested compounds. b) The detectability of DNA breakage within telomeres by the Comet-assay is limited by the size of DNA fragments resolvable by this technique (10-800 kb) (13): As estimates of telomere lengths in human peripheral leukocytes vary between 5 and 15 kb (33), fragments of these segments will be detected only in exceptional cases and cannot be differentiated from breaks in telomere-adjacent DNA.

In conclusion, the highly important usefulness of Comet-assay for estimating drug sensitivity in transformed (malignant) cells (9-11,34-39) has been confirmed by the present comparative experiments which found clearly differing responses to the examined mutagens of the different cell types. Of particular interest, however, is the exemplary finding that specific DNA probes can now detect and quantitatively estimate the involve-ment of specific genomic regions in the genetic damage induced by therapeutically applied mutagens. This may be useful in oncogenetics in general and for prognosis of malignant disease of specific genetic outfit, if amplified DNA sequences will be addressed by these examinations in future.

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