# Radiation sensitivity of lymphocytes from healthy individuals and cancer patients as measured by the comet assay

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**Abstract** Lymphocytes of healthy volunteers (*n*=24) and of tumour patients (*n*=30, 18 of whom had experienced severe side-effects) were irradiated with x-rays in vitro. DNA damage was analysed after 0.25–2 Gy and DNA repair after 2 Gy, and quantification of both endpoints was done by the comet assay. The individual differences in radiation-induced DNA damage as well as in the repair kinetics were observed to be striking for both healthy donors and tumour patients. After a repair time of 3 h, following 2 Gy x-irradiation, some of the healthy volunteers showed no residual DNA damage at all in their lymphocytes, whereas others revealed about 30%. There was no indication that our results were affected by either age, gender or smoking habits. Slow repair kinetics and high amounts of residual damage were characteristic for many but not all tumour patients who had experienced severe side-effects in their normal tissues during or after radiotherapy (*n*=18). Our conclusion is that those individuals showing poor DNA repair characteristics in the lymphocytes following in vitro irradiation, have a high probability of being radiosensitive. The opposite conclusion is not necessarily true: if repair is effective, this does not mean that the individual is radioresistant, because factors other than impaired repair may cause radiosensitivity.

## Introduction

We know that individuals show marked differences in radiation sensitivity (e.g. [1, 2, 3]), which has, of course, considerable consequences in the fields of both radiation protection and radiation therapy. In the case of a radiation accident it is not the physical dose alone that will decide the outcome for the exposed person, but also the individual radiation sensitivity. Dose limits for occupational exposure are based on an "average person" from a larger population implying that for some individuals the current dose limits pose a higher risk than for others. In radiation therapy, the knowledge of individual radiation sensitivity enables the therapist to apply either higher or lower tumour doses than is usually done; in the former case, this will improve local tumour control (without harming the normal tissue) and in the latter case will avoid severe side-effects in the normal tissue.

Up to now, there has been no fast and reliable assay system that could be used to quantitatively determine the differences in individual radiation sensitivity. Since DNA repair definitely has a considerable impact on radiation sensitivity, we decided to check whether the comet assay might give useful information in this context.

The comet assay determines the amount of DNA damage in individual cells by applying a weak electric field to the nuclear material, from which almost all compounds except DNA have been removed (for detailed information on the assay see [4, 5, 6, 7, 8]). This initial DNA damage is measured immediately after radiation exposure, whereas the effect of repair processes is determined for various time periods (in the case of lymphocytes usually up to 3 h) after incubation of the cells at 37°C.

In the experiments presented here, we compared initial DNA damage and DNA repair in the lymphocytes of healthy people after radiation exposure in vitro. In addition, we determined the same endpoints in the lymphocytes of radiotherapy patients who had reacted with severe side-effects in their normal tissue, as well as in the lymphocytes of tumour patients who had not undergone any therapy. For this analysis we used the original comet assay version of Ostling and Johanson [4] with only minor modifications. The advantage of this assay is its high sensitivity in comparison to the alkaline or neutral versions and it evidently not only detects strand breaks, but also changes in the DNA conformation [9]. This suggestion of Östling and Johanson is supported by the data of Klaude et al. [10].

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We found marked differences in lymphocyte response of healthy people. This was true for the initial damage as well as for the repair kinetics and the residual damage after 3 h of repair. Many of those patients that reacted with severe side-effects to radiation therapy, still showed an extraordinarily high amount of residual damage after 3 h of repair.

## Materials and methods

### Comet assay

The method applied in this study has been described in detail by Böcker et al. [11]. Only those aspects that are necessary for the understanding of the results presented here are outlined.

Unstimulated lymphocytes were isolated from heparinised blood samples using the Ficoll-Hypaque technique [12]. Radiation exposure in vitro was carried out by a Stabilipan x-ray machine (Siemens, Erlangen, Germany) at 240 kVp, a dose rate of 1 Gy/min, and a copper filtration of 0.5 mm. The cells were suspended in warm agarose (0.75%) for several seconds and distributed on a microscope slide that had been precovered with agarose (0.1%). After gelling at 4°C, the cells were lysed in an SDS solution (pH 9.5) for 15 min at 18°C. After washing in distilled water at 18°C, electrophoresis was carried out at pH 8.4, 2.5 V/cm, and 16 mA for 4 min at 10°C. The electrophoresis conditions were chosen in such a way, that just a very small tail appeared in unexposed DNA, which ensures that small additional effects by, e.g. radiation, are detected. The slides were washed again, stained with propidium iodide and analysed by image analysis [13]. The ratio of the DNA intensity in the tail to that in the head region of the comet, was used as a measure of DNA damage. Each data point was based on at least 40 comets.

### Radiotherapy patients

We analysed blood samples from various tumour patients who were treated in the Clinic for Radiation Therapy of Essen University Hospital. Ethical clearance and informed consent was obtained for and from all patients. The tumours were of very heterogeneous origin, including nasopharynx, oropharynx, tonsil, hypopharynx, oesophagus, lung, breast, cervix and prostate. More details of these analyses together with additional cases will be given in a future publication to address the possibilities of the use of the comet assay in radiation therapy. Blood was analysed from 12 patients (median age: 57 years, range: 45–77) immediately before the start of radiation therapy. In 18 cases, we used blood samples from patients (median age: 56 years, range: 32–78) who reacted unexpectedly to radiation either during or early after therapy (14 cases within the first 90 days after start of radiation therapy) or late after therapy (4 cases later than 90 days after start of radiation therapy). Classification of the side-effects was according to Sauer [14]; class 3 and, in particular, class 4 effects were considered to be severe. The group of healthy donors consisted of 24 individuals (median age: 50 years, range: 24–75).

### Endpoints and statistics

DNA damage was determined in the range of 0.25–2 Gy and DNA repair after exposure to 2 Gy between 15 and 180 min. The curves obtained for damage and repair were quantified by two approaches: either by just one data point (in the case of damage the reaction to 2 Gy, in the case of repair the residual damage after 180 min of repair) or by the information obtained from the entire curve, i.e. from the area under the curve (AuC, calculated by adding up the areas of the trapezia of the respective curves after connecting the data points by straight lines).

All statistical analyses were run using the GraphPad Prism software (version 2.01). Statistical significance was calculated by the two-sided, unpaired Student's *t*-test.

## Results

### Initial DNA damage

Initial radiation-induced DNA damage was determined in such a way that repair is excluded. This is achieved by keeping the cells on ice during irradiation and at 18°C during the first steps of lysis (2.5% SDS buffer, pH 9.5; lower temperatures cause precipitation of SDS; any repair activities are quickly stopped under these lysis conditions). Figure 1 demonstrates that marked differences can be observed when the initial radiation-induced DNA damage is analysed in lymphocytes of different healthy people and this is particularly true for doses up to 1 Gy. The reduction in variability observed after 2 Gy is primarily due to the fact that at doses exceeding 2 Gy, only a marginal additional increase in the amount of DNA in the tail of the

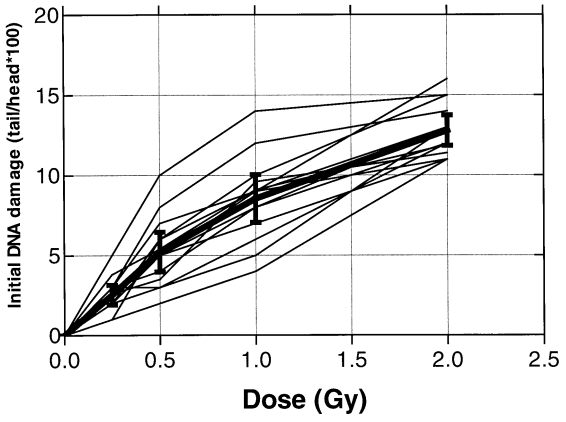


**Fig. 1** Initial DNA damage in lymphocytes of 14 healthy donors (*Bold line* arithmetic mean with 95% confidence intervals)

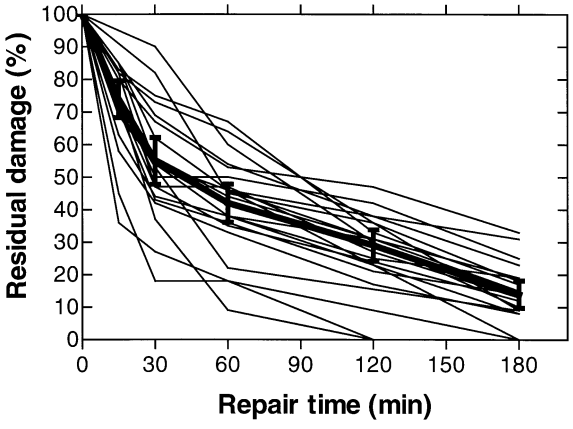


**Fig. 2** Repair kinetics in lymphocytes of 24 healthy donors (*Bold line* arithmetic mean with 95% confidence intervals; the data have been normalised for differences in the initial amount of damage)

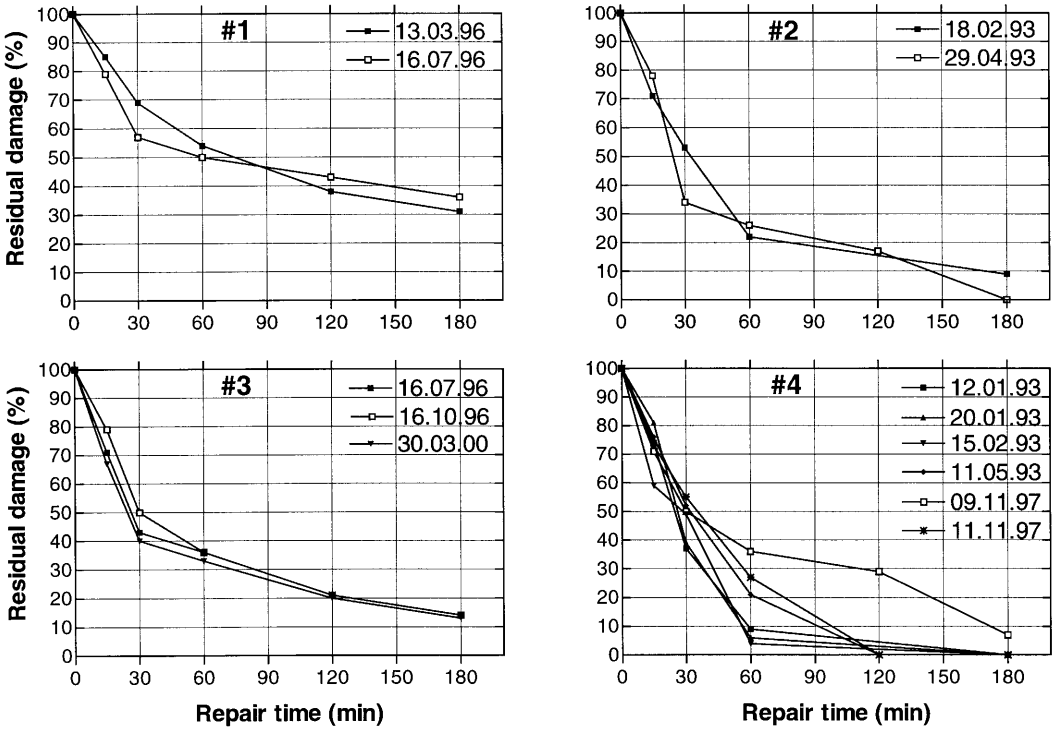


**Fig. 3** Repair kinetics in lymphocytes of four individuals analysed at different time points (The data have been normalised for differences in the initial amount of damage)

comet is obtained under our experimental conditions. This levelling-off obviously already starts above 1 Gy.

## Repair kinetics

In contrast to the determination of initial DNA damage, repair studies are run at 37°C in the incubator for various time intervals. Figure 2 shows the pronounced variability of the shape of repair kinetics of lymphocytes of healthy individuals after a dose of 2 Gy. Obviously, the cells of quite a high number of healthy donors showed a rather slow speed of repair and a remarkably high amount of residual damage. In those cases in which we analysed longer repair times (up to 360 min and in one case up to 24 h), only small amounts of the residual damage seen after 180 min were removed.

One could suspect the method itself to be very unreliable resulting in the wide scatter that can be seen in Fig. 2, but this is not the case. When checking the reproducibility of the analyses by measuring the same slide repeatedly, very similar results were obtained [13]. There is additional evidence for the high reproducibility of the assay. For four individuals, two or more analyses of the same person at different times are presented in Fig. 3. Donor 1 shows a slow speed of repair and considerable residual damage, donors 2 and 3 are intermediate, whereas donor 4 reveals a very effective repair. A most interesting aspect is that, with the exception of one data point (120 min of the 09.11.97 curve) of donor 4 (this data point was inconspicuous, when remeasured just 2 days later), the repair kinetics are very similar when measured for the same person at intervals of some days, months, or years.

Figure 4 indicates a weak relationship between the amount of initial damage and repair capacity. Figure 4a shows the area under the curve (AuC) that has been determined. High values point to much damage with regard to initial DNA damage, whereas, in the case of DNA repair, high values point to poor repair characteristics. It appears as if those individuals whose lymphocytes show severe initial damage, also have a poor repair system; this trend, however, is too weak to achieve statistical significance (i.e. the slope is not significantly different from zero; $P>0.1$). It should be kept in mind, however, that in those lymphocytes with a high amount of initial damage, many more DNA lesions have to be repaired than in lymphocytes with low numbers of DNA lesions, and, hence, require a longer repair time.

Figure 4b reveals that there is some relationship between the amount of damage after exposure to 2 Gy and the percentage of residual damage after 3 h of repair: individuals with low initial damage after 2 Gy tend to show smaller amounts of residual damage expressed as a percentage of the initial damage ($P<0.1$).

No statistically significant differences were observed among age groups below and above the median age (of about 50 years), between smokers and non-smokers (either never smokers or non-smokers for at least the past 5 years), or between males and females, when various endpoints were analysed (Table 1). However, one has to be cautious in drawing final conclusions, because the numbers of females as well as of smokers were low in our analysis. This was also the major reason for us to re-

**Table 1** Effects of age, gender, and smoking on DNA damage and DNA repair (mean ± standard deviation are shown)

| Factor | DNA damage | | DNA repair | |
|---|---|---|---|---|
| | Damage at 2 Gy | AuC | Residual damage after 3 h | AuC |
| Age | | | | |
| <Median (50 years) | 12.6±1.8 (12) | 16.8±3.6 (7) | 12%±9% (12) | 70.4±19.0 (11) |
| >Median | 13.3±1.9 (12) | 14.0±2.7 (7) | 15%±9% (12) | 69.2±22.0 (11) |
| Gender | | | | |
| Female | 12.5±0.6 (4) | 13.9±2.6 (2) | 18%±11% (4) | 71.0±23.4 (4) |
| Male | 12.9±2.0 (20) | 15.7±3.7 (11) | 12%±11% (20) | 69.5±19.4 (19) |
| Smoking habit | | | | |
| Non-smoker | 13.1±1.9 (12) | 16.4±3.5 (8) | 15%±12% (12) | 71.8±20.1 (10) |
| Smoker | 13.0±1.7 (5) | 15.0±2.6 (3) | 15%±6% (5) | 74.8±19.2 (4) |

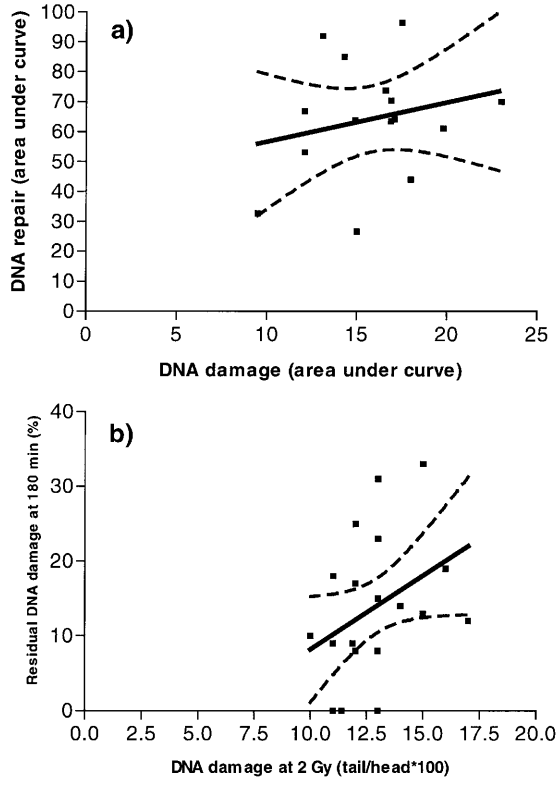


**Fig. 4** **a** Relationship between initial DNA damage and DNA repair quantified by the area under curve. **b** Relationship between DNA damage at 2 Gy and residual damage at 180 min after radiation exposure to 2 Gy (Regression line and 95% confidence intervals)

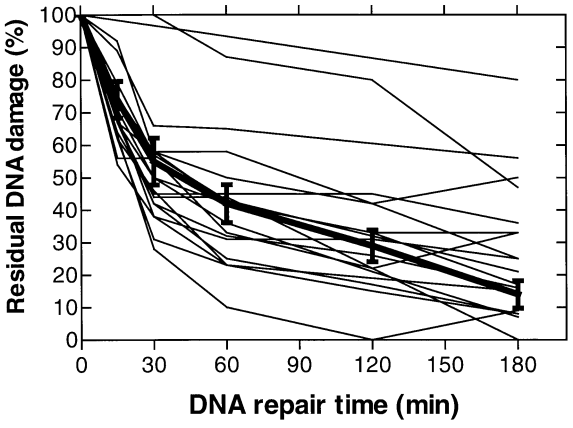


**Fig. 5** Repair kinetics in lymphocytes of 18 tumour patients reacting with severe side-effects in normal tissues under or after radiation therapy (The bold line shows the mean reaction and the 95% confidence intervals of the 24 healthy donors mentioned in Fig. 2 and does not represent the mean reaction of the 18 tumour patients; the data have been normalised for differences in the initial amount of damage)

frain from analysing the even smaller patient group accordingly; in addition, we have the problem in the patient group that the response was more heterogeneous than for healthy donors.

Figure 5 shows the individual repair kinetics referring to the lymphocytes of 18 tumour patients who responded with either acute (e.g. dry or moist desquamation, lymphoedema, mucositis, dysphagia) or late (e.g. ulcer, fibrosis) severe side-effects during or after radiotherapy. The primary aim of this paper is to point out that there is some evidence that the various repair kinetics in healthy individuals are related to an individual level of radiosensitivity. Therefore, we do not consider any further clinical aspects here, but reserve them for a future publication dealing with the clinical points of view. The spectrum of the repair kinetics of the patients with severe side-effects is evidently much broader than that of the healthy donors (compare Figs. 2 and 5).

The comet distribution of single cells proved to be much more informative than the mean values. Due to the fact that the presentation of data would be very space-consuming, only one example is given here (see Fig. 6). A healthy donor with a very effective repair has been compared with a patient who had severe skin damage after 10 fractions of 2 Gy each (corresponding to a maximum skin dose of 10 Gy) for radiation treatment of an oesophagus carcinoma. The amount of initial damage (observed under the same experimental conditions) and of damage after irradiation with 2 Gy in vitro is similar in both individuals, but repair after 3 h is considerably different. Most of the patients lymphocytes were unable to restore the DNA, whereas the lymphocytes of the healthy donor showed the same distribution of comets as before radiation.

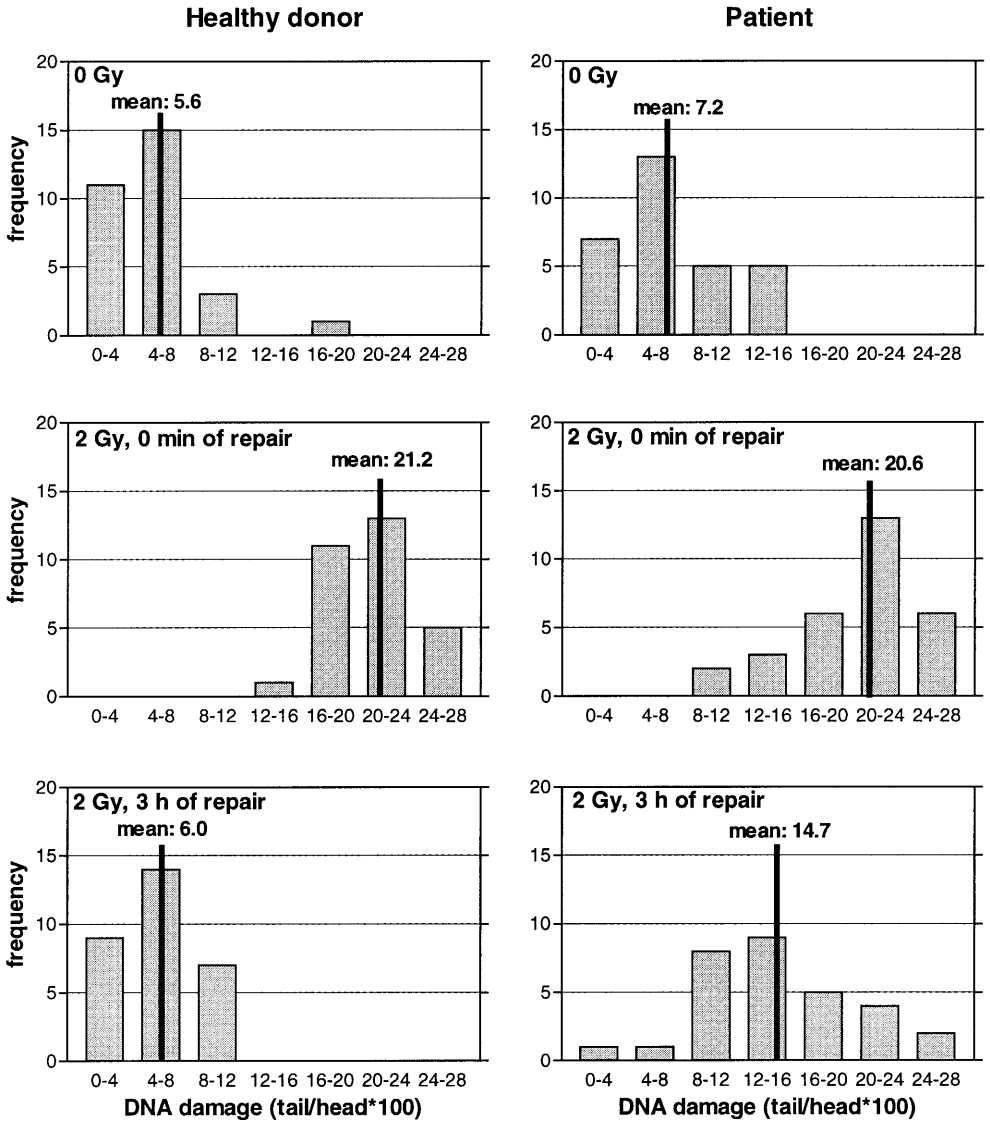


**Fig. 6** Comparison of comet distribution in lymphocytes of a healthy donor and a patient reacting with severe side-effects (skin damage after 10 fractions of 2 Gy, corresponding to a maximum skin dose of 10 Gy) in unexposed and 2 Gy (in vitro) exposed cells after 0 and 180 min of repair

Comparing the dose response curves of healthy individuals and of tumour patients reacting with severe side-effects to radiation therapy, reveals that there are no significant differences up to 120 min of repair, but that the differences are statistically significant for the residual DNA damage after 180 min of repair ($P<0.05$; Fig. 7). Figure 7 also demonstrates that this difference is not due to the fact that these patients have a tumour, because 12 tumour patients analysed before radiation therapy were not different from the control individuals with respect to the repair kinetics of their lymphocytes. Likewise, combined radio-/chemotherapy does not bias the results, because those two patients in the group with severe side-effects who received a combined radio-/chemotherapy until immediately before the comets were analysed were not particularly conspicuous: one of them showed an average response, the second a markedly better repair characteristic than the average of this group of 18 over-reactors. Of course, our population is far too small to form

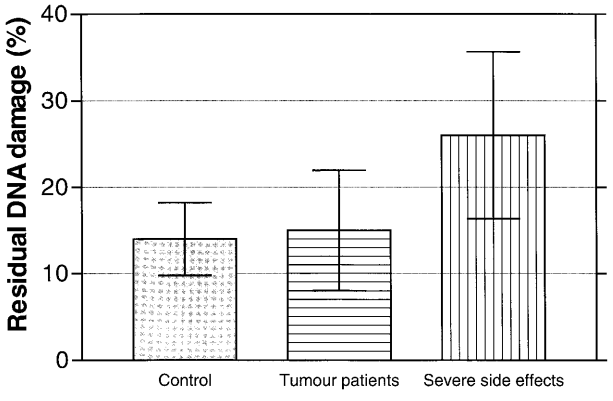


**Fig. 7** Comparison of the residual damage after 3 h of repair after radiation exposure (2 Gy x-rays) of lymphocytes of controls ($n$=24), tumour patients before radiotherapy ($n$=12), and tumour patients reacting with severe side-effects under or after radiotherapy ($n$=18) (The error bars represent the 95% confidence intervals)

any conclusions with regard to the impact of a chemotherapy on the results, but the data at least are not biased by chemotherapy in the direction of poor repair characteristics.

## Discussion

Our main objective is to provide evidence that the observed individual differences in the repair kinetics of lymphocytes after an in vitro radiation exposure to 2 Gy, may provide valuable information with regard to individual levels of radiosensitivity.

First of all, we were able to demonstrate that there are pronounced differences in the lymphocyte response of different individuals as to the amount of initial DNA damage (i.e. without any repair), to the time dependence of repair, and, in particular, to the amount of residual damage after 3 h of DNA repair. To observe differences in the induction of initial DNA damage is scientifically interesting, but without much relevance for radiation risk, because what counts in this context is the amount of damage that is not or only very slowly repaired. One reason for differences in the amount of initial damage might be related to the differences in the fraction of lymphocyte subpopulations with different degrees of chromatin condensation. This is a characteristic that can affect the amount of initial DNA damage [15]. We will restrict ourselves to a discussion of the relevance of repair.

An essential prerequisite of using the comet assay for the assessment of individual radiosensitivity is its reproducibility. Many experiments in the past have shown that strictly standardised experimental conditions (temperature at the various experimental steps, buffer pH, agarose concentrations) are required to achieve this goal [16]. Figure 3 reveals that when choosing those standard conditions, specific DNA repair kinetics are characteristic for an individual. In other words, a person responding with a fast complete repair does this repeatedly over a longer period, and the same is true for the opposite (slow incomplete repair) as well as for intermediate situations. It is, of course, conceivable that sometimes there may be intra-individual variability due to specific factors (e.g. diseases, medication, hormonal fluctuation), however, intra-individual variability appears by and large to be low.

The studies of the repair kinetics in lymphocytes of patients reacting with severe side-effects strongly support the comet assay to be a useful indicator for individual radiosensitivity. This is particularly true for the analysis of residual damage after 3 h of repair. One could assume that the on the average, higher amounts of residual damage in lymphocytes of the patients is, to some extent related to the radiotherapy itself. This effect, however, cannot be very pronounced, because the 4 patients in our population showing side-effects 8, 15, 16, and 23 years after therapy, had the same higher amount of residual damage as those patients who responded with severe side-effects during or soon after radiotherapy. In the meantime, we have analysed 50 additional patients prior to radiotherapy and after 5 and 10 fractions of 2 Gy and, on average, did not find differences in the repair kinetics before and during radiotherapy (results to be published).

There are other publications also reporting individual differences of the repair characteristics as determined by the comet assay (e.g. [17, 18]), but it is difficult to compare the results directly, because different assay protocols were used. In all studies, however, endpoints were detected that showed large inter-individual differences and the authors concluded that these differences might be useful for the quantification of individual radiation sensitivity.

The comet assay alone will, of course, not be able to give complete information on individual radiosensitivity, because too many complications have to be considered such as:

1. The comet assay does not reveal anything about the fidelity of repair
2. There are other factors (other than repair) affecting radiosensitivity
3. Lymphocytes may not be able to adequately reflect any specific radiosensitivity of various organs or the whole organism
4. There may be confounders (such as certain drugs) which could interfere.

However, the comet assay applied to lymphocytes may have the function of a warning signal when markedly poor repair kinetics are detected, because there is a high probability that the individual is radiosensitive.

## Conclusions

Measuring DNA repair in lymphocytes by the comet assay alone, will not completely describe individual levels of radiosensitivity. Thus, it is not surprising that not all tumour patients who reacted with severe side-effects to radiation therapy are conspicuous in the comet assay. Therefore, the comet assay seems to be useful as one system in a battery of assay systems. One should be very careful, however, when a poor repair characteristic has been identified by the comet assay, because then there seems to be a high probability that this individual is radiosensitive. On the other hand, one should not feel safe, when the comet assay signals a normal repair characteristic, because factors other than repair may be responsible for an increased radiosensitivity.

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